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Biology of and Novel Therapeutic Approaches for Epithelial Cancers of the Aerodigestive Tract

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Biology of and Novel Therapeutic Approaches for Epithelial Cancers of the Aerodigestive Tract

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Jack A. Roth, M.D.
Waun K. Hong, M.D.
James L. Mulshine, M.D.

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PREFACE

With the recent publication of the report from Peto et al (*Lancet* 339:1268–1278, 1992), the staggering mortality of cigarette smoking comes into full focus. The premature world-wide mortality from tobacco consumption over the next decade will exceed by an order of magnitude the aggregate mortality of the Bubonic plague over the entire 14th century. The enthusiasm for the declining frequency of smoking in this country must be tempered by two facts: First, we are the dominant nation responsible for international tobacco commerce. Second, any reduction in tobacco-related mortality will lag for several decades behind positive trends in smoking cessation.

The potential for benefit of early detection and intervention research is greatest for carcinogen-exposed populations. Against this backdrop emerges a sense of urgency in considering the state of our scientific and medical knowledge for a principal cause of smoking-related mortality, that is cancer of the upper aerodigestive tract. With this Monograph we explore the current understanding of the basic science of the early carcinogenesis for the organs that form the first target of tobacco exposure. A complementary publication was published as a *Cancer Research* Supplement (52:2635s–2770s,1992) reviewing the opportunities for translating the basic science information into initial steps for developing effective early detection and intervention approaches principally for lung cancer. Together these publications give us a snapshot of the formidable challenge ahead in moving to a prevention-oriented approach to epithelial cancer control.

We thank the staff of the *Journal of the National Cancer Institute*, especially Edwin Haugh, Julia Redmond, and Daniel Ihde for their support in making this document possible. We thank Ann Rodgers and Noel Smith of the Biomarkers and Prevention Branch for their help in coordinating the review process, which was an effort to ensure the rigor of this important subject matter.

Capturing the proceedings of this workshop as a monograph was an effort to stimulate greater translational research activity in this critical area. As is evident with even cursory consideration of this document, enormous amounts of work remain to be done if we are to have the tools to begin implementation of a comprehensive prevention-oriented cancer control program for these inconceivably lethal diseases.

Introduction

Jack A. Roth,[1] Waun Ki Hong,[2] James L. Mulshine[3,4]

Cancers of the aerodigestive tract, including lung, head and neck, and esophagus, are among the most common tumors. These may serve as models for human carcinogen-induced cancers. Cancers of these three sites are frequently associated with exposure to tobacco carcinogens, and patients frequently develop primary cancers at multiple sites, suggesting field effects as postulated by Slaughter on carcinogen-exposed upper aerodigestive epithelium. The results of conventional treatment are poor for this group of cancers, and long-term survival has changed little over the last 5 years. Thus, a potentially positive new direction is to follow the admonition of Dr. Michael Sporn and attempt to treat the process of carcinogenesis prior to the development of an invasive cancer. This new approach entails defining parameters that comprise carcinogenesis. This can be best accomplished by exploring the biology of field cancerization, using a model of the cigarette-smoke-exposed upper aerodigestive epithelium. The focus of this monograph is to explore the biology of the aerodigestive system as it relates to cancer in an effort to elucidate relevant field cancerization dynamics. These insights could lead to new strategies for early detection and rational biochemical intervention with epithelial neoplasias of these three related sites.

Researchers have made scientific advances in understanding carcinogenesis of these tumors at the molecular level. These insights will provide a rational basis for the design of prevention and therapy strategies and will identify molecules that have prognostic significance. Recent studies have implicated dominant oncogenes (myc and ras families, erbB2), tumor suppressor genes (Rb, p53), and growth factors (GRP, TGF-α, IGF-I) in the genesis of these tumors. New findings relevant to multidrug resistance may have important applications for these cancers. However, the interrelationships of these mechanisms are poorly understood.

This issue presents the proceedings of a conference, Biology of and Novel Therapeutic Approaches for Epithelial Cancers of the Aerodigestive Tract, held in Steamboat Springs, Colo., April 1–7, 1991, and sponsored by the Keystone Symposia on Molecular and Cellular Biology. A major goal of this meeting was to bring together investigators from multiple disciplines to synthesize recent data into molecular models of tumor genesis and progression. It was hoped that observations in one area or cancer type would cross-fertilize research in a related area. Diagnostic, prognostic, and therapeutic applications of these studies were discussed.

Identification of the molecular events leading to the development of lung cancer may identify new targets for therapy. Drs. Slebos and Rodenhuis discuss ras mutations in lung cancer. Dr. Roth and coworkers describe molecular events in non-small-cell lung cancer and strategies to alter oncogene expression. Dr. Johnson and coworkers studied amplification of the myc gene family in tumors from patients with small-cell lung cancer. Dr. Birrer and coworkers describe the role of c-jun in tumor promotion.

Mechanisms for control of growth and differentiation are discussed by several participants. Growth factors and their receptors have been identified on lung cancer cells. Drs. Nicolson, Cavanaugh, and Inoue have identified lung paracrine growth factors that can stimulate the growth of cells metastasizing to the lung. Dr. Lee and coworkers show that antibodies to the epidermal growth factor receptor can inhibit the growth of lung cancer cells. Clinical studies using anti-epidermal growth factor receptor monoclonal antibodies are presented by Dr. Mendelsohn. Dr. Lotan and coworkers studied the effects of retinoic acid on the receptor for epidermal growth factor. Drs. Jetten, Nervi, and Vollberg discuss type I transglutaminase in the program of squamous differentiation in human epidermal and tracheobronchial epithelial cells. Dr. Rice and coworkers present studies on differentiation markers in keratinocytes. Dr. Carey and coworkers describe a β_4 integrin that is a prognostic marker for head and neck cancers. The clinical studies supporting this are presented by Drs. Wolf and Carey. Dr. Linnoila and coworkers describe a surfactant-associated protein that identifies a distinct group of lung cancers. Monoclonal antibodies to tumor markers may detect classes of antigens that are overexpressed in bronchial epithelial cells recovered in sputum, thereby detecting the presence of a lung cancer earlier than conventional histology. Data in support of this concept are shown by Dr. Mulshine.

Both primary prevention and chemoprevention strategies have the potential to reduce mortality from aerodigestive tract cancers. Dr. Greenwald summarizes past work and future directions in this area. Drs. Slaga and Gimenez-Conti describe a hamster cheek pouch model for studying events in the genesis of premalignancy and

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malignancy of oral cancer. Drs. Moon, Rao, and Detrisac present trials of chemopreventive agents in a hamster carcinogenesis model for the tracheobronchial tree and lung. Drs. Garewal, Sampliner, and Fennerty discuss chemopreventive studies in patients with Barrett's epithelium. Dr. Meyskens discusses the important issue of biomarkers to be used as intermediate endpoints for chemoprevention studies. Drs. Lippman and Hong summarize the current status of chemoprevention trials.

Drs. Vitolo, Letessier, Johnson, and Whiteside characterize immune effector cells from the tumor and lymph nodes of head and neck cancer patients. Drs. Yang, Grimm, and Roth report clinical immunotherapy trials showing that immunotherapy can mediate regression of metastatic lung cancer. Dr. Gazdar and coworkers analyze mechanisms that may contribute to drug resistance in lung cancer.

Drs. Eipper, Green, and Mains discuss expression of processing enzymes central to the final bioactivation of many neuropeptides associated with lung cancer cells. Dr. Battey and coworkers describe cloning of the GRP receptor. Dr. Coy discusses synthesis of competitive antagonists of GRP. Drs. Treston, Mulshine, and Cuttitta review regulation of peptide hormone processing by tumor cells and relate how these pathways may be a strategic target for a new class of intervention approaches. Dr. Bunn and coworkers present studies on the responsiveness of lung cancer cells to neuropeptides.

The conference summarized the current status of our knowledge of the biology of human lung cancer. Our challenge for the future will be to expand this knowledge base and translate it into improvements in prevention, diagnosis, and treatment.

We would like to dedicate this monograph to the many pioneers who have made the current work and bright prospects possible. This type of integrated tumor biology leading to an improved patient management approach is a concept first pioneered by the founders of such seminal groups as the International Association for Lung Cancer, the Lung Cancer Study Group, and the National Cancer Institute-Veterans Administration Oncology Branch. Although clinical research has not resulted in dramatic improvement in current patient outcome, it has permitted extensive studies illuminating the basis of cancer progression events that now provide the leads for developing prevention-based approaches to these devastating malignancies.

The conference organizers gratefully acknowledge the generous educational grants in support of this program from Berlex Biosciences, Inc. (sponsor of the meeting), Bristol-Myers Oncology Division, and Abbott Laboratories.

Role of Biology and Prevention in Aerodigestive Tract Cancers

Peter Greenwald[1], Harriet R. Stern[2,3]

ABSTRACT—Primary prevention aimed at smoking control and chemoprevention for high-risk persons or patients at risk for a second cancer provide strong potential for cancer prevention and control of aerodigestive cancers. The National Cancer Institute (NCI) has a major effort to build this area of research. The Third Upper Aerodigestive Tract Cancer Task Force Workshop, held in 1989 under the auspices of the National Cancer Institute's Organ System Program, reviewed the opportunities for chemoprevention research on aerodigestive epithelial cancers such as the regulation of growth and differentiation in normal and malignant cells. The chemoprevention program's drug development effort is evaluating several promising candidate agents for future clinical testing and the NCI clinical intervention program is supporting several trials of selected chemoprevention agents with demonstrated potential for inhibiting cancers of the lung, bronchus, oral cavity, and esophagus. Of special interest to this program is the assessment of beta-carotene, retinol and related synthetic retinoids, and several vitamin and mineral combinations under study in high-risk international populations. Chemoprevention in the medical setting is a major focus of NCI's Community Oncology Program (CCOP), a network designed not only to increase accrual of patients to trials but also to speed adoption of state-of-the-art therapies. Public health strategies are directed toward control of exposure to tobacco. The focal point for these activities is NCI's Smoking, Tobacco, and Cancer Program (STCP). STCP smoking cessation efforts are targeted at specific populations that are at greater risk for developing cancer including youth, minority and ethnic groups, women, smokeless tobacco users, and heavy smokers. Two of the world's largest controlled intervention trials conducted by the STCP are underway: the Community Intervention Trial for Smoking Cessation (COMMITT), which focuses on 6.5 million heavy smokers in 11 pairs of matched communities in North America, and the American Stop Smoking Intervention Study (ASSIST), a coalition model designed to reach millions of Americans through existing health promoting systems. [J Natl Cancer Inst Monogr 13:3–14, 1992]

Aerodigestive tract cancers of the head and neck, oral cavity, pharynx, and larynx were estimated in 1990 to account for 43 000 new cases and 13 000 deaths (SEER); the figures for other aerodigestive tract cancers of the esophagus, lung, and bronchus are expected to account for nearly one third of the 500 000 cancer deaths predicted for 1990. Epidemiologic data indicate that the worldwide incidence of these malignancies is increasing. Table 1 shows the age-adjusted incidence and mortality rates in the United States by aerodigestive site for the years 1984–1988 (*1*). Despite concerted efforts in surgery, radiotherapy, and chemotherapy to control aerodigestive cancers, 5-year survival rates in the United States have improved only marginally since 1974 for whites and less for blacks, as shown in Table 2 (*1*).

Several years following successful primary therapy of early-stage or locally advanced head and neck tumors, 30% to 50% of patients may have local or regional recurrence, 20% to 30% may have distant metastasis, and 10% to 40% may have a second primary tumor (*2*). A major cause of death following primary therapy is from the development of a second tumor. These discouraging facts and the identification of specific risk factors for these cancers have focused attention on the strong potential for cancer prevention and control of aerodigestive cancers. The anatomic distribution of second malignancies of the aerodigestive tract reflects the phenomenon of "field carcinogenesis," a concept put forth in the classic report by Slaughter et al on oral cancer (*3*). "Field carcinogenesis" is demonstrated in tissue samples from different sites within the aerodigestive tract that can simultaneously reflect different stages of carcinogenesis from histologically and clinically normal tissue to malignant tissue. The development of second primary epithelial tumors in regions of the head and neck, lung, and esophagus may be due to diffuse mucosal initiation and continued promotion by exogenous carcinogenic factors. These initiating or promotional factors include immoderate tobacco and alcohol use typically found in patients with these cancers; these agents may in fact be synergistic (*4–7*).

CLINICAL EVIDENCE FOR FIELD CARCINOGENESIS

In patients with regionally advanced head and neck cancer, the patterns of relapse after complete remission following therapy are well documented. Combined modality therapy with chemotherapy, surgery, and/or radiation did not reduce the incidence of local recurrences or distant metastases in a study of 103 patients with previously untreated squamous cell carcinoma of the head and neck (*8*).

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Table 1. Age-adjusted incidence and mortality rates by site, sex, and time period*

	All races					
	SEER incidence (1984–1988)			SEER mortality (1984–1988)		
Site	Total	Male	Female	Total	Male	Female
All sites	372.5	434.1	335.4	166.8	209.1	138.5
Oral cavity and pharynx	11.1	16.8	6.5	3.2	4.9	1.9
Lip	1.2	2.5	0.3	0.0	0.0	0.0
Tongue	2.2	3.2	1.4	0.7	1.1	0.5
Salivary gland	1.0	1.2	0.8	0.2	0.3	0.2
Floor of mouth	1.2	1.8	0.7	0.2	0.3	0.1
Gum and other mouth	2.0	2.5	1.5	0.5	0.7	0.3
Nasopharynx	0.6	0.9	0.4	0.3	0.4	0.2
Tonsil	1.1	1.6	0.6	0.3	0.5	0.2
Oropharynx	0.3	0.5	0.2	0.2	0.3	0.1
Hypopharynx	1.1	2.0	0.5	0.3	0.5	0.1
Other buccal cavity and pharynx	0.4	0.6	0.3	0.5	0.8	0.3
Digestive system	79.8	99.7	64.9	42.0	53.9	33.3
Esophagus	3.8	6.2	1.9	3.4	5.7	1.6
Respiratory system	63.4	94.8	39.6	45.3	69.1	27.7
Nose, nasal cavity, and middle ear	0.6	0.8	0.5	0.2	0.2	0.1
Larynx	4.6	8.4	1.6	1.2	2.3	0.5
Lung and bronchus	57.2	84.0	37.1	43.5	66.0	27.0
Pleura	0.7	1.3	0.2	0.2	0.3	0.1
Trachea, mediastinum, and other respiratory organs	0.3	0.3	0.2	0.1	0.2	0.1

*Incidence and mortality rates are per 100 000 and are age adjusted to the 1970 U.S. standard population.

Patients with early-stage head and neck cancer are at significant risk for second primary tumors, since their risk for competing causes of death such as metastic disease or local relapse is greatly diminished by their primary therapy.

A study by McDonald et al of a retrospective survey of 235 patients with laryngeal cancer who were followed for a median 10 years reported that 21% of these patients developed secondary malignant tumors (SMTs) (*9*). Sixty-two percent of the SMTs occurred in the aerodigestive tract; 18% occurred in the head and neck; and 4% occurred in the lung. It is ironic that SMTs occurred most often in patients diagnosed with early-stage disease (*9*). Cooper and colleagues, in a report from the Radiation Therapy Oncology Group study of 928 patients who received radiotherapy, observed that 25% of patients who were followed for 8 years developed SMTs. Nearly 60% of SMTs occurred in the aerodigestive tract, as in the McDonald study (*5*). Again, of clinical importance was the finding that the highest risk for developing SMTs occurred in patients diagnosed at the earliest disease stage. This probably reflects the shorter survival and higher death rates from the primary tumor among patients with advanced-state disease.

The concept of "field carcinogenesis" has implications for chemoprevention research, which is described later. It is notable that increases in mortality rates among patients with head and neck cancer are attributable to the second primary tumor. Moreover, there appears to be a distinct targeting of the lung, the head and neck region, and the esophagus as sites for these second tumors. Five-year survival rates from the second regional tumor are very low, suggesting the need for effective strategies in addition to surgical, radiotherapy, or chemotherapy treatments (*10*). In the selection of chemopreventive agents, "field carcinogenesis" suggests that particular attention be given to suppressing promotion rather than blocking initiation.

PREVENTION AND CONTROL: NCI EFFORTS

During the past several years, new approaches have been developed to lessen the burden of head and neck cancers. The Third Upper Aerodigestive Tract Cancer Task Force Workshop held in 1989 under the auspices of the National Cancer Institute's (NCI) Organ System Program focused on the rapidly expanding area of chemoprevention research on aerodigestive epithelial cancers. These workshops are designed to enhance interactions between basic science and clinical investigators and expedite advances in the area of cancer research. The Third Upper Aerodigestive Tract Cancer Task Force Workshop focused on the progress and opportunities for regulation of growth and differentiation in normal and malignant cells of the upper aerodigestive tract and the chemoprevention of these cancers (*4*).

HIGHLIGHTS OF CHEMOPREVENTION RESEARCH

The NCI Chemoprevention Program

The focal points for NCI's disease prevention and control activities related to aerodigestive cancers are two ma-

Table 2. Five-year relative survival rates (%) by selected sites, white and black males and females

Site	Year of diagnosis				
	1960–1963*	1970–1973*	1974–1976†	1977–1980†	1981–1987†
White males and females					
All sites	39	43	50.0	50.5	52.5‡
Oral cavity and pharynx	45	43	54.8	54.2	53.6
Esophagus	4	4	5.1	5.8	8.9‡
Larynx	53	62	66.1	67.2	69.9
Lung and bronchus	8	10	12.3	13.3	13.4‡
Black males and females					
All sites	27	31	38.6	38.8	38.4
Oral cavity and pharynx	—§	—	35.3	34.3	31.2
Esophagus	1	4	3.9	3.5	6.2‡
Larynx	—	—	58.6	58.4	54.3
Lung and bronchus	5	7	11.3	11.6	10.8

*Rates are based on End Results Group data from a series of hospital registries and one population-based registry.

†Rates are from the SEER Program. They are based on data from population-based registries in Connecticut, New Mexico, Utah, Iowa, Hawaii, Atlanta, Detroit, Seattle-Puget Sound, and San Francisco-Oakland. Rates are based on follow-up of patients through 1986.

‡The difference in rates between 1974–1976 and 1981–1987 is statistically significant ($P < .05$).

§— = Valid survival rate could not be calculated.

jor program efforts: the Smoking, Tobacco, and Cancer Program and the Chemoprevention Program. The National Cancer Institute established the Laboratory of Chemoprevention in the 1970s as part of its support of this research area. A major benefit from these early investigations conducted in the Laboratory of Chemoprevention has been the extensive developmental research on the effects of retinoids in suppressing malignant transformation. In 1982, the work on the chemoprevention of the carcinogenic process was extended under the NCI Chemoprevention Program to accomplish the following: identify and characterize new agents with proven efficacy in preventing carcinogenesis in animals and with a high probability of preventing cancer in humans; conduct efficacy and toxicity testing of candidate compounds in animal systems; and conduct clinical trials with candidate agents that potentially may suppress tumorigenesis. To accomplish this agenda, the NCI Chemoprevention Program has developed a strategic system for identifying and testing potential chemopreventive agents. Defined criteria and decision points for the continued evaluation of promising candidates are based on agent efficacy, toxicity, tolerance, and safety in humans and provide the framework for the strategy shown in Fig. 1.

The Research Evidence

The scientific rationale relevant to the chemoprevention of aerodigestive cancers has been based initially on findings reported in epidemiologic and laboratory studies. The ability of vitamin A and its synthetic analogues, the retinoids, to inhibit growth and induce differentiation in malignant cell lines was first reported in 1925—a historic milestone. Retinoids can suppress carcinogenesis in a variety of epithelial tissues, including the skin, trachea, lung, and oral mucosa. A number of retinoids are active in vitro and in animal models in squamous cell carcinoma of the head and neck (*11*). In mice, retinoids inhibit the activity of ornithine decarboxylase, an enzyme associated with tumor promotion processes (*12*). Retinoids also block cell transformation through inhibition of polypeptide transforming factors, such as sarcoma growth factor (*13*). More recently, Lotan et al showed that retinoic acid suppressed epidermal growth factor (EGF) binding, which may be responsible for retinoid in vitro and in vivo growth-inhibitory effects on head and neck cancers (*14*).

In human epidemiologic studies, the risk of developing head and neck, esophageal, and lung cancer is reported to be inversely related to dietary consumption of vitamin A and/or carotenoids. For example, two large prospective studies that evaluated vitamin A intake and the risk of lung cancer have yielded consistent findings. In a study of 8278 Norwegian men, an inverse relation was found between lung cancer risk and vitamin A intake, an observation that was confirmed in a study update (*15,16*). The same result was found in a 10-year study of more than 250 000 Japanese adults (*17*). In a smaller study, bronchogenic carcinoma incidence was inversely correlated with carotenoid intake in 2100 American male smokers (*18*).

Carotenoids, dietary precursors of vitamin A that are widely present in plant foods, are of interest as chemopreventive agents for aerodigestive cancers. A review of prospective and retrospective studies indicates that low intakes of vegetables and fruits are consistently associated with an increased risk of lung cancer (*19*). Although only a few laboratory studies have explored the chemopreventive effects of carotenoids in animals, a direct chemopreventive role for beta-carotene has been suggested because of its very efficient ability to deactivate singlet oxygen and trap organic free radicals (*20*). The extensive study of beta-carotene is in part based on the experimental evidence demonstrating its antitumorigenic

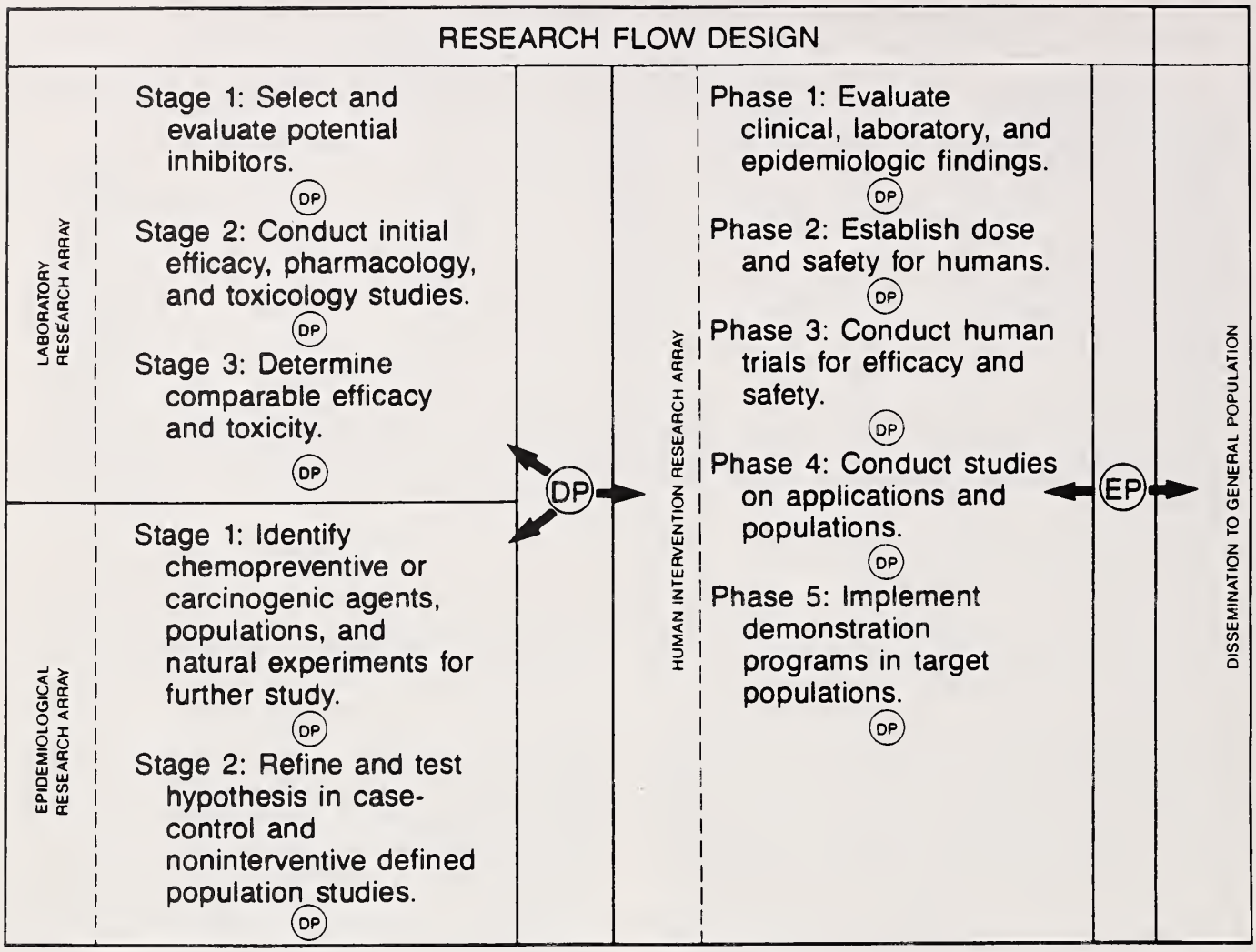

Fig 1. NCI chemoprevention program.

effect, low toxicity, and immunostimulating activity against epithelial cancers. Beta-carotene seems to play the most important role; however, the importance of other carotenoids is not known.

Results from retrospective and prospective studies correlating serum retinol levels and relative cancer risk are inconclusive. These findings reflect the facts that serum retinol is not directly influenced by dietary intake and varies only slightly among populations with normal vitamin A status. Also, serum retinol levels may not accurately reflect local retinoid concentration in some tissues (*21*).

Other nutrients for which protective roles in aerodigestive cancers have been suggested include vitamin E (alpha-tocopherol) and vitamin C (ascorbic acid), although these findings are not entirely consistent (*22*). For example, in a study of serum vitamins A and E, beta-carotene, selenium, and the risk of lung cancer, Menkes et al found that low levels of serum vitamin E increased the risk of developing any type of lung cancer, and low levels of serum vitamin A increased the risk of developing squamous cell carcinoma of the lung (*23*), a result that differs from an earlier report by Willett et al in which no significant association was detected between cancer risk at all sites and serum levels of total carotenoids or vitamin E (*24*).

Other epidemiologic studies show low levels of molybdenum exposure via food and/or drinking water to be associated with an increased rate of esophageal cancer. Sodium molybdate has demonstrated chemopreventive activity in several animal experiments, with notable effects in the rat esophagus (*22*). Deficiencies in dietary selenium may also play a role in the development of esophageal cancer (*25*). A case-control study conducted from 1983 to 1985 in Hawaii indicated a clear negative association between beta-carotene intake and lung cancer risk (*26*). The fact that vitamin C, along with vitamin A and beta-carotene, is present in many fruits and vegetables limits the ability of epidemiologic studies alone to distinguish the specific dietary factors responsible for apparent benefits. However, these findings provide the scientific basis for identifying and characterizing potential chemopreventive

agents in doses that exceed usual nutrient allowances for healthy people.

Chemopreventive Agent Development

The program to identify and develop for human use chemical agents that have the potential for inhibiting the progression of cancer covers every aspect of agent development from initial literature searches to phase III clinical trials. The elements of the program are information management; biochemical and in vitro screening, with 169 compounds currently undergoing testing; the in vivo animal model screens, with 107 compounds now being tested; drug procurement; toxicity testing, with 14 compounds being tested; and clinical trials currently representing almost 40 studies. Many are natural products, with some currently available pharmaceuticals also included (anti-inflammatory steroids, hormones, and prostaglandin synthetase inhibitors). These compounds are structurally diverse and express multiple biologic and toxic effects. In humans, retinoids have been most effective therapeutically in the prevention of skin diseases; however, nondermatologic premalignancies such as oral leukoplakia, bronchial metaplasia, laryngeal papillomatosis, and several urogenital malignancies also respond to retinoid therapy. Several derivatives of retinoic acid with considerably less toxicity than the parent compound have been synthesized, including three undergoing clinical evaluation: 13-*cis*-retinoic acid (isotretinoin, Accutane), etretinate (Tegison), and 4-hydroxyphenylretinamide (4-HPR, Fenretinide).

Preclinical Research

Preclinical investigations identify and evaluate promising candidate substances to determine their efficacy, safety, and pharmacologic parameters using in vitro and in vivo screening systems. If a particular chemopreventive agent or intervention satisfies all of the laboratory and epidemiologic decision points, an evaluation is made as to whether the strength of the preclinical research data justifies intervention in humans.

In vitro test systems exposed to a number of carcinogenic substances evaluate the potential chemopreventive activity of new agents in cell and tissue models. These models include cell transformation bioassays, growth bioassays, combination agent screening, and modulation of intermediate preneoplastic biologic markers. In vivo screening studies focus on providing the initial evidence of chemopreventive positive findings in animal models, dose-response data, evidence of agent acceptability and tolerance, and the initial evidence of agent toxicity. Assays evaluating the efficacy and toxicity of agents include short-term studies, lifetime studies in rodents, and multigenerational studies. Thus far there appears to be adequate correlation between in vivo and in vitro screens. The preclinical screening studies, it should be noted, can only clarify chemopreventive potential prior to confirmation of positive findings in clinical settings (*27*).

New Chemopreventive Agents That Show Promise

Antioxidants, anti-inflammatory agents, inhibitors of cell proliferation, and agents that counteract prostaglandin, ornithine decarboxylase, and other initiating or promoting tumor activity are under development. Several agents appear particularly promising for cancers of the head and neck.

Difluoromethylornithine (DFMO). DFMO has demonstrated experimental tumor inhibition at several sites and is now under intensive evaluation in humans. DFMO, an inhibitor of ornithine decarboxylase, an essential key enzyme for the maintenance of cell growth and functions, is being tested for its effectiveness against small-cell lung cancer alone and in combination therapy (*28*). A 1-year chronic toxicity study has recently been completed, and DFMO has entered a phase I chemoprevention study to evaluate the agent's antitumor drug effectiveness.

4-Hydroxyphenylretinamide (4-HPR)/other retinoids. 4-HPR has demonstrated a spectrum of chemopreventive activity in animal models. It is currently under evaluation in a phase III clinical trial for the prevention of primary lesions of the contralateral breast in women who have had breast cancer. There may be a potential for this agent in the treatment of aerodigestive tract cancers. 4-HPR preclinical screening is underway for lung cancer prevention (*28*). A short-term trial for the prevention of oral cancer with high-dose isotretinoin has led to a longer-term study of isotretinoin administered in combination with beta-carotene in an attempt to prolong remission of the precancerous lesion, leukoplakia, and lessen toxic effects.

***N*-Acetylcysteine.** *N*-acetylcysteine (NAC) has been successfully tested for preclinical toxicity, and a phase I clinical trial is now being conducted. First used as a mucolytic drug in patients with bronchitis, NAC is now reported to have a chemopreventive effect on urethane-induced lung tumors in mice. Based on a successful completion of the phase I trial, NAC appears to be a candidate for a phase II study for high-risk lung cancer populations (*28*).

CLINICAL INTERVENTION

Compounds found to have high efficacy and low toxicity in the preclinical tests are evaluated in phase I and II human clinical trials using intermediate endpoint markers in a limited number of healthy subjects who are usually healthy but are at high risk for preneoplastic lesions.

Intermediate Endpoint Marker Studies

A variety of intermediate endpoints, or biologic markers, of precancerous conditions, which reflect not the evidence of but the potential for neoplastic progression, have become available for testing and may be used to identify or evaluate risk modulation by chemopreventive agents in high-risk target populations. These phase I and II studies, usually with a limited number of subjects, are using a variety of techniques that make possible the excellent experimental models demonstrating the inhibition of malig-

nant cell transformation before the onset of neoplastic change. For example, by systemically administering agents such as the retinoids, 13-*cis*-retinoic acid and retinyl acetate, beta-carotene, and vitamin E, it has been possible to demonstrate the inhibition of the precancerous oral lesion, leukoplakia. The chronic peptic ulcer of the esophagus first observed by NR Barrett and subsequently referred to as "Barrett's epithelium" is associated with an increased risk of esophageal cancer. This premalignant model demonstrates increased ornithine decarboxylase activity when dysplastic changes are present. Both the premalignant lesion, Barrett's epithelium, and ODC, the biochemical indicator, are presently being evaluated in intermediate marker studies. Bronchial micronuclei are reported by other investigators as a possible intermediate endpoint marker for detecting tobacco-initiated tracheobronchial carcinogenesis that appears to be a biologic predecessor of squamous-cell lung cancer (*29*). Table 3 lists potential intermediate endpoint studies for oral and lung cancer sites. These endpoint markers, when proved sensitive, specific, and quantifiable, may be useful techniques for improving the reliability and feasibility of clinical chemoprevention trials.

First reported in the literature about 10 years ago, early results using the modulation of intermediate endpoints have been encouraging, although much work remains before their reliability can be validated. For example, the synthetic retinoid etretinate was tested for its ability to reduce bronchial squamous cell metaplasia (*30*). The trial, conducted in a high-risk population (heavy smokers), indicated that the severity of bronchial metaplasia was reduced in treated subjects. In a further study, treatment with vitamin A, beta-carotene, or 4,4′-diketo-beta-carotene was evaluated among betel nut/tobacco chewers, a population at high risk for oral cancer. The study assessed the effects of treatment on the occurrence of micronuclei (a genotoxic marker) in exfoliated oral epithelial cells. Both vitamin A and beta-carotene notably inhibited the occurrence of micronucleated epithelial cells, but 4,4′-diketo-beta-carotene, which is not converted to vitamin A, showed no effect (*31*).

Other clinical trials using retinoid therapy and marker endpoints in head and neck cancer prevention research include a study by Hong et al, a double-blind, placebo-controlled study of oral premalignant lesions. This study demonstrated that 13-*cis*-retinoic acid could cause temporary remission of leukoplakia and reverse dysplasia. The patients in this study had a positive response rate of 67% in the treated group and only 10% in the placebo group. However, over 50% of the responding patients relapsed within 2 to 3 months after the completion of therapy (*32*).

A highlight of more recent clinical chemopreventive research is the report of the prevention of second primary tumors with the retinoid isotretinoin at the M.D. Anderson Cancer Center. Prospectively studied were 103 randomized patients who were disease free after primary treatment for squamous-cell cancers of the larynx, pharynx, or oral cavity and who received 50 to 100 mg/m^2 of body surface per day of isotretinoin or placebo for 12 months. The results strongly indicate that daily treatment with high doses of isotretinoin is effective in preventing second primary tumors in patients who have been treated for squamous-cell carcinoma of the head and neck, although isotretinoin did not affect the failure rate for local, regional, and distant recurrences of the original tumor (*2*).

THE NCI CLINICAL INTERVENTION PROGRAM

Positive results from various preclinical studies of candidate agents must be ultimately tested in an actual clinical setting. Most of the trials are phase I or II. More than 30 human intervention trials sponsored by the NCI Chemoprevention Program are currently in progress. The trials are testing selected chemopreventive agents with demonstrated potential for inhibiting cancers of the lung, bronchus, oral cavity, and esophagus, as well as cancers of the breast, colon, skin, and cervix. In the case of aerodigestive cancers, particular attention is being directed toward beta-carotene, retinol and related synthetic retinoids, vitamins C and E, molybdate, dithiolthiones, and DFMO (*28*). Of special interest to this program are the development and testing of combinations of chemopreventives that have enhanced efficacy and that are given in lower dosage to decrease toxicity. Some promising combinations are being evaluated for various organ systems.

The significant suppression of second primary tumors achieved with isotretinoin provides impetus for designing more studies of primary and adjuvant chemoprevention with retinoid and other agents in subjects at high risk for aerodigestive cancers. Of the many other cancer prevention trials in progress, 17 are examining the effects of beta-carotene and retinoids, alone or in combination with other agents, on cancer incidence. For example, a Boston study of 22 000 healthy male physicians, ages 40 to 84, is evaluating the influence of beta-carotene and aspirin on both total cancer incidence and cardiovascular disease.

Table 3. Potential intermediate endpoint studies*

Site	Agent	Population	Endpoint
Lung	Dithiolthiones, DFMO, molybdate	Smokers	Squamous metaplasia/dysplasia, micronuclei, epithelial growth factor, oncogene expression, quantitative DNA analysis, ornithine decarboxylase
Oral	4-HPR, dithiolthiones, dehydroepiandrosterone	Leukoplakia	Metaplasia/dysplasia, micronuclei

*Adapted from (*28*).

The aspirin arm of the study is completed, and results will be forthcoming from the effect of beta-carotene supplementation on reducing the risk of several cancers. Other cancer sites being studied for the effects of retinoids and/or carotenoids include the skin, lung, oral cavity, cervix, colon, and breast. Table 4 summarizes data on those trials currently being conducted in the United States for aerodigestive cancers and includes information on study population, site, and agent dosage.

International Studies

One large-scale study, the Alpha-Tocopherol, Beta-Carotene Lung Cancer Prevention Study, is being conducted by NCI in collaboration with the National Public Health Institute of Finland. The alliance offers a unique opportunity to do research among a high-risk Finnish population, which has marginal per capita intakes of several micronutrients and among the highest incidence of lung cancer in the world. These high lung cancer rates, which have also been attributed to cigarette smoking, make Finland a desirable target for a lung cancer prevention strategy. Oral administration of beta-carotene and alpha-tocopherol is being tested in a population of 29 000 males, ages 50 to 69, who are heavy smokers. This randomized, placebo-controlled, double-blind study has four separate treatment groups being evaluated, using a 2 × 2 factorial design. Incident cancers will be identified through chest x ray during intervals of the intervention and at the conclusion of the 6-year clinical follow-up procedures. A reduction in cancer incidence as demonstrated in the trial will be compared with national trends by monitoring Finland's government-operated health registers.

NCI is also collaborating with the Cancer Institute of the Chinese Academy of Medical Sciences in two randomized, double-blind, 5-year placebo-controlled intervention trials in Linxian, China. This area has a particularly high incidence of esophageal cancer. Nutrition assessment data show that nutritional status could be improved by vitamin supplementation. One study, the General Population Trial, is assessing the effects of multiple vitamin-mineral combinations on the incidence of esophageal cancer among a test population of 30 000 men and women. As shown in Fig. 2, there are eight intervention groups (i.e., fractional factorial design) receiving placebos or specified combinations of vitamin A, beta-carotene, zinc, riboflavin, niacin, vitamin C, molybdenum, selenium, and vitamin E at doses one to four times the U.S. Recommended Dietary Allowance. The second study, the Dysplasia Trial, will examine the effects of multiple vitamins/minerals on a high-risk population with severe esophageal dysplasia. In a simple study design, 3400 men and women, ages 40 to 69, will receive either a placebo or a multiple vitamin/mineral supplement at levels one to four times the Recommended Dietary Allowance. Participants will be evaluated for disease progression or regression. In both studies, cancer incidence and mortality will be determined through hospital record pathology slides and radiographic reviews in conjunction with the Linxian Esophageal Cancer Registry.

Future Prospects

Although there has been enormous progress in chemoprevention research with the retinoids, this class of compounds is believed to offer prospects for other significant advances. The synthesis of new generations of retinoids and the exploration of their structure–activity relationships are therefore continuing under NCI support. A number of other promising chemopreventive agents have been identified and are being evaluated in preclinical and phase I and II clinical trials. The chemopreventive agent development program is an essential part of seeking Food and Drug Administration (FDA) approval for human testing, and the toxicity and safety assessments require extended time periods for obtaining results. Because of the large commitment of resources, the selection of agents, the recruitment of patients, and other study parameters are critical. A major effort at NCI is to encourage small, efficient studies using biologic endpoints.

Table 4. Chemoprevention branch clinical trials:* aerodigestive cancers

Study title	Study site, population	Agent, dosage, and schedule
Trial of beta-carotene and lung cancer in high-risk women	Lung High-risk women	Beta-carotene, 50 mg QOD Vitamin E, 600 mg QOD
Chemoprevention of lung cancer with retinoids/beta-carotene	Lung Cigarette smokers (five study sites)	Beta-carotene, 30 mg/day Retinoids, 25,000 IU/day
Beta-carotene and lung cancer chemoprevention	Lung Men, exposed to asbestos	Retinol, 25,000 IU/QOD Beta-carotene, 50 mg/day
A randomized trial of aspirin and beta-carotene in U.S. physicians	All sites Male physicians	Beta-carotene, 50 mg/alternate days Aspirin, 325/mg alternate days
Chemoprevention of human bronchial metaplasia/dysplasia	Lung Chronic smokers	13-*cis*-retinoic acid, 1.0 mg/kg daily
Chemoprevention of human premalignant oral lesions	Oral cavity Oral leukoplakia	13-*cis*-retinoic acid, 1.5 mg/kg for 3 months, then 0.5 mg/kg daily for 9 months
Bowman-Birk inhibitor (BBI) and oral cancer	Oral cavity Oral leukoplakia	BBI

*Placebo controlled.

Intervention Groups

Placebo*	A	B	AB*
C	AC*	BC*	ABC
D	AD*	BD*	ABD
CD*	ACD	BCD	ABCD*

Fig 2. The Linxian esophageal cancer prevention trial is a fractional factorial design based on a 2^4 factorial. The asterisks indicate the eight intervention groups included in a half-replicate of the 2^4 factorial design. Thirty thousand participants, 40 to 69 years old, receive either a daily placebo or one of the seven vitamin/mineral combinations. Treatment agents are (A) vitamin A, beta-carotene, zinc; (B) riboflavin, niacin; (C) vitamin C, molybdenum; (D) selenium, vitamin E.

Intermediate markers used in clinical trial protocols are a valuable adjunct that may provide a more complete understanding of the carcinogenic process than do the results of cancer endpoint protocol designs.

Public Health Policy and Prevention Research

Recent advances in genetic and molecular biology may allow asymptomatic individuals at risk for common cancers such as lung cancer to be identified before the cancer progresses to malignancy. Specifically, laboratory investigations over the last 50 years have demonstrated that retinoids, such as isotretinoin, are potent inhibitors of cellular proliferation. The significant suppression of second primary tumors with isotretinoin, achieved by Hong and colleagues (*8*), provides the basis for designing studies of primary and adjuvant chemoprevention with retinoids and related agents in subjects at high risk for aerodigestive cancers. These and other research opportunities will be competing for resources. Policies that enhance collaboration between clinical and experimental investigations are important adjuncts to research environments.

Social Responsibility of Government in the Public Interest

Prevention research, like all research, is undertaken when the requisite skills and resources are available to accomplish the research at the highest possible level of excellence, especially when findings may have direct implications for public policy or for the well-being of subjects. In the effort to achieve this level of excellence, progress may be slowed. Scientific research has therefore become a collective undertaking requiring large investments and constant support by government and universities (*33*).

When a research strategy is developed and implemented, one cannot be sure of the findings until the work is largely completed. This is somewhat analogous to funding large clinical trials where the outcome is not known for several years. The speed and efficiency with which we are able to translate resulting scientific knowledge into health practices for the community has become increasingly important for the prevention and treatment of cancer.

The NCI's Community Clinical Oncology Program (CCOP), a network of more than 1000 community physicians participating with their patients in NCI-approved research, is an example of a network designed not only to increase accrual of patients to trials but also to act as a mechanism to increase adoption of state-of-the-art cancer therapies (*34*). Chemoprevention research in the medical setting is a high priority of the CCOP. Examples of CCOP prevention and control protocols include 1) a double-blind trial to test the effects of low-dose 13-*cis*-retinoic acid on the prevention of second primary tumors in stage I and II head and neck cancers and 2) a pilot trial of alpha-tocopherol in oral leukoplakia.

The rationale for the program is based on the fact that more than 80% of cancer patients are treated in the community and on evidence indicating that physicians who provide care for cancer patients in the community environment can maintain high-quality clinical research activities similar to those in academic centers. The program also exemplifies an efficient model for technology transfer and continuing medical education in cancer, as well as an excellent resource for cancer prevention and control studies. In cancer treatment, the availability of clinical trials for a wider segment of cancer patients and adoption of the results of those trials are the best chances for immediate reduction in cancer mortality.

Cost-Benefit Analysis

Cancer care is becoming more complex and expensive. At the same time there is growing concern about controlling overall health care costs without compromising access and quality of care. More knowledge about the financial burden of health care and about the clinical efficacy of medical procedures is needed. These concerns form the basis of NCI's interest in understanding patterns of care and cost related to cancer prevention, control, and treatment.

Generally, when a cancer therapy is found to be effective, there are two sources of savings: the savings due to the delay in terminal care associated with death and the savings due to avoided treatment for recurrent cases of cancer. Cancer prevention—through the administration of specific chemical regimens, chemoprevention—provides a broad potential for reducing cancer incidence and mortality rates.

PREVENTION AND CONTROL: TOBACCO USE

The publication of four landmark retrospective studies 40 years ago on the patterns of smoking among lung cancer patients firmly established the link between tobacco use and lung cancer (*35, 36*). Tobacco initiates a multistep carcinogenic process in the upper aerodigestive tract and lung, beginning with the development of the premalignant metaplastic and dysplastic states and progressing to cancer (*37*). The higher frequency of premalignant and malignant oral lesions recently observed among young Americans

because of their increasing use of smokeless tobacco is also a concern. Thousands of smoking studies—including eight prospective and more than 50 retrospective investigations, referred to in the 1964 and subsequent Surgeon General reports—have consistently reported an association between tobacco smoking and lung cancer. Lung cancer rates in white males have been declining since 1977 and among black males since 1982. Nearly 800 000 deaths were postponed or prevented between 1964 and 1985 because Americans either quit or did not start smoking (*38*). Subsequent studies that followed this report established smoking as a major cause of cancers of the larynx, oral cavity, and esophagus. Cigarette smoking is estimated as the primary cause of 70% of the increase in cancer of the larnyx from 1950 to 1985 (*38*).

A major effort of NCI has been directed toward developing effective intervention strategies to control the use of exposure to the etiologic carcinogens found in tobacco. The focal point for NCI's disease prevention and health promotion research activities related to tobacco use and cancer is the STCP, whose aim is to decrease the incidence and mortality of cancers caused by or related to tobacco use (*38*).

Selected Smoking Cessation Trials

STCP smoking cessation strategies are directed at developing intervention methods in the following areas: school-based interventions, self-help techniques, physician/dentist-initiated interventions, mass media interventions, and community-based interventions. Specific populations that are at greater risk for developing cancer and/or are amenable to prevention strategies are a major target for STCP interventions and include youth, minority ethnic groups, women, smokeless tobacco users, and heavy smokers. Also being addressed is research on diffusion methods directed at delivering proven intervention techniques that have the potential to achieve a broad public health benefit for large segments of the U.S. population.

From 1984 to 1989, STCP planned controlled intervention trials to develop the most effective national strategies to reduce cancer mortality. This included the Community Intervention Trial for Smoking Cessation (COMMIT), which focuses on the testing of a community-based intervention protocol that can be disseminated nationwide to meet NCI's Year 2000 objectives to reduce smoking prevalence. This trial involves more than 6.5 million people, either directly or indirectly, making it one of the largest smoking intervention trials in the world. Because of their increased risk for cancer and their difficulty in quitting, heavy smokers (25 or more cigarettes a day) are the emphasis of COMMIT. These heavy smokers account for nearly half of all the lung and smoking-related cancers among smokers yet represent only one fourth of all smokers.

As a community trial, COMMIT requires the implementation of a complex intervention protocol in partnership with diverse community organizations and groups. The trial design includes 11 pairs of communities in North America that are matched in size, demographics, and location. The COMMIT interventions will build on, coordinate, and facilitate community smoking control activities. The overall goals of the trial are to:

- Increase the priority of smoking as a public health issue
- Improve the community's ability to modify smoking behavior
- Increase the influence of existing policy and economic factors that discourage smoking
- Decrease social norms and values that support smoking

As large and comprehensive as the STCP trials were, in 1987 NCI officials recognized that they would have little impact on smoking behavior and even less on cancer mortality rates unless their methods were applied on a national scale. As a resut, in April 1987, NCI convened a 3-day meeting of over 250 scientists and public health experts to plan the next step in the continuum to reduce smoking prevalence and related cancers. The expert consultants strongly recommended reaching 50 million Americans by using community-based tobacco control coalitions to be established in entire states or in large metropolitan areas. This recommendation is the basis for a large-scale demonstration project—ASSIST/2000, the American Stop Smoking Intervention Study—that will widely apply tested strategies.

Regardless of population, any state is eligible as an ASSIST site, as are large metropolitan areas with a population of at least 2 million. Formation of community-based tobacco control coalitions that will be responsible for developing, coordinating, and implementing comprehensive tobacco prevention and control plans is required by each ASSIST site. Because of their overall responsibility for the public's health, state and local health departments will serve as the fiscal agents for the coalitions. The strengths of the ASSIST coalition model include 1) the ability of member organizations to expand smoking control activities through existing systems and 2) its support of comprehensive smoking control policies such as increasing restrictions on smoking in public places and limiting sales and promotions of tobacco to minors.

ASSIST will be implemented in two phases. During phase I, each funded coalition will perform site analysis by surveying its region to determine the status of tobacco control. Each coalition will develop a comprehensive tobacco prevention and control plan reflecting the unique needs of each community. Phase II will involve carrying out detailed action steps developed during the planning process. These activities will include training health care professionals to provide counseling services and targeted interventions for high-risk populations.

Examples of Smoking Cessation Techniques

Smoking cessation methods are often divided into two broad areas, unassisted and assisted. Examples of unassisted methods of cessation include quitting "cold turkey"; gradually decreasing the number of cigarettes smoked per day; using low-tar or low-nicotine cigarettes; quitting with friends, relatives, or acquaintances; using special cigarette filters or holders; or using other nonprescription products. Assisted methods of cessation include

attending a course or a program cost free or for a fee; consulting a psychiatrist or psychologist; using hypnosis or acupuncture; or chewing nicotine gum. Because nicotine gum requires a prescription, and physicians are typically urged to provide cessation counseling with the gum, this method is considered an assisted method with high potential for success (*39*).

Adolescent School-Based Smoking Prevention

Since 1984, the STCP has supported 23 intervention trials aimed at adolescents (*38*). Smokeless tobacco is the focus of seven of these trials; one trial uses media techniques to reach youth; and the other 15 trials focus either partially or entirely on prevention of adolescent smoking through school-based programs. These trials may be categorized into trials developing new curriculums or techniques for smoking prevention (eight trials); trials adapting curriculums that already exist for smoking prevention to more current approaches or special populations (five trials); and trials conducting long-term follow-up of youth who were exposed, as long as 10 years ago, to smoking prevention programs in the school (two trials) (*40*).

Physician and Dentist Interventions

The STCP has supported the involvement of physicians and dentists in five major smoking cessation intervention trials for the public. These trials directly or indirectly affect more than 100 000 patients and 6000 physicians and dentists. The settings for these trials include private practices, public clinics, health maintenance organizations, and residency training programs. The techniques used involve one or more of the following: nicotine gum, role playing, use of videotapes, relapse prevention techniques, development of office-based reminder systems, training for office staff, and self-help strategies and booklets to increase physician and dentist compliance in using the cessation protocols.

Self-Help/Minimal Interventions

Nearly all types of cessation interventions (e.g., those carried out solely by the individual or delivered by physicians or other health professionals through the media, group programs, or worksite programs) depend to some extent on the use of self-guided materials or strategies. Beginning in 1984, seven STCP self-help/minimal intervention trials were initiated, which have directly or indirectly involved 3.5 million people. The two objectives of the STCP self-help/minimal intervention components are 1) to develop *primary* self-help materials and strategies for individuals who wish to quit smoking on their own and 2) to provide *supplementary* materials for use in programs that require interaction with trained individuals but to some extent also depend on self-help interventions.

Minority Intervention Targets

Among blacks, the prevalence of cigarette smoking and tobacco-related cancers, particularly those of the lung, esophagus, and larynx, is higher than among other U.S. subpopulations or among whites. In the past 20 years, limited efforts to reduce smoking rates of black Americans have been undertaken; however, few programs have approached this problem in a culturally relevant manner. As a result, between 1985 and 1987, NCI began supporting six smoking intervention trials targeted at black Americans, with the objective to develop, implement, and evaluate programs focused on reducing the smoking prevalence of this group.

The first objective of these trials is to better understand the smoking habits, smoking knowledge, quitting motivation, quitting attempts, and quitting experiences of black smokers and exsmokers. The development of cost-effective smoking prevention and cessation programs specifically oriented toward the needs and concerns of black smokers or blacks at risk to begin smoking is the second objective. These trials are being conducted in diverse geographic regions and in a variety of social settings, including schools, community health clinics, businesses, community action programs, and, in some cases, entire communities.

Prevalence of smoking among Hispanic males is slightly in excess of white males, and smoking among Hispanic females, although not as prevalent as among white females, has risen. The prevalence of both male and female Hispanic adolescents who smoke closely parallels that of the general adolescent population. Based on this, it can be predicted that tobacco-related cancer rates among Hispanics in the next century will be very similar to those of whites.

In response to this profile of Hispanic smokers, in 1986 STCP began supporting four large intervention trials directed at the prevention and cessation of tobacco use among Hispanic-Americans. These trials include several Hispanic subgroups, use Spanish-language material appropriate to the various Hispanic groups represented in the trials, and use a variety of channels to reach this population, including Spanish-language media, community outreach, and Hispanic organizations.

Women Smoker Interventions

Twenty-seven percent of the American female population smoked in 1987, a 21% decrease since 1966, when U.S. female smoking rates reached their highest level at 34% (*38*). Although encouraging, this decline is in sharp contrast with the 40% reduction in American male smoking rates seen over the same period. Women may have greater difficulty in quitting cigarettes than do men, as suggested by some survey and experimental data (*38*). Fear of postcessation weight gain, dependence on social support as an aid to quitting, and smoking as a response to multiple-role stress experienced by women today are examples of various social, psychologic, and behavioral explanations for this increased difficulty.

Roughly half of the population reached by STCP's 60 intervention trials are women. STCP has funded five projects since 1985 that focus solely on smoking cessation in women. These five projects either focus on a particular

population of female smokers or target the specific needs and concerns related to cessation that are unique to women.

With more than 150 000 tobacco-related deaths expected to occur in the United States annually, the Smoking, Tobacco, and Cancer Program under the direction of NCI has become the largest effort of its kind in the world. Total program funding from 1982 through 1990 is expected to exceed $240 million. Successful program delivery has shown a quantifiable favorable effect on lung cancer morbidity and mortality with trends for lung cancer significantly decreased in males under age 55 (*1*). The decrease in smoking prevalence in these groups fostered by NCI programs is a priority with the National Cancer Institute.

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Molecular Approaches to Prevention and Therapy of Aerodigestive Tract Cancers[1]

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ABSTRACT—We describe studies defining several molecular events in human non-small-cell lung cancer (NSCLC). These include increased growth factor and growth factor receptor expression and oncogene alterations. The epidermal growth factor receptor (EGFR) and erbB2 are expressed by NSCLC cells. Transforming growth factor-alpha (TGF-α) is produced by NSCLC and may mediate autocrine growth stimulation. Specific inhibition of K-ras oncogene expression by an antisense K-ras construct reduces the growth rate and tumorigenicity of NSCLC cells. Studies with antisense p53 in NSCLC with a homozygous p53 mutation suggest that the presence of the mutant form contributes to the transformed state. [J Natl Cancer Inst Monogr 13:15–21, 1992]

Lung cancer is one of the few human cancers where the causative agent is known. However, knowledge of the molecular events resulting in development and progression of malignancy in the lung is recent and incomplete. Definition of the type and sequence of molecular changes in the cell exposed to tobacco carcinogens can aid in the rational development of prevention and treatment strategies.

It is likely that there is both a heritable genetic predisposition to lung cancer and sporadic somatic genetic alterations that occur as a result of prolonged exposure to tobacco carcinogens (*1–4*). In this paper, we will discuss findings in the cell and molecular biology of non-small-cell lung cancer (NSCLC) that may be relevant to the development of new prevention and treatment strategies.

Small-cell lung cancer (SCLC) has been widely studied because of the availability of established SCLC cell lines (*5–8*). Twenty percent of lung cancers are of the SCLC type, and their biologic behavior is different from the more common NSCLC. SCLC metastasizes early in its course and shows sensitivity to chemotherapy, followed by early recurrence. NSCLC grows locally in a significant number of patients and does not respond well to chemotherapy. Differences in molecular events will be described and therefore, molecular mechanisms may not be generalizable among the different forms of lung cancer.

The major categories of genetic events implicated in lung cancer include growth factor receptor expression, autocrine growth factor secretion, dominant oncogene activation, and deletion of tumor suppressor genes. The relative importance and sequence dependence of these in the genesis and progression of lung cancer is unknown. Our laboratory has conducted a series of studies to find the contribution of these factors to the development and progression of aerodigestive cancers including NSCLC and esophageal cancer. This paper will summarize studies showing that 1) expression of erbB2 may contribute to the development of NSCLC, 2) TGF-α can function as an autocrine growth factor for NSCLC, 3) specific reversal of mutated K-ras expression can alter NSCLC growth rate and tumorigenicity, and 4) the presence of the mutant p53 gene contributes to the transformed state.

MATERIALS AND METHODS

Techniques that are non-standard or that are modified are described.

Cell Lines and Culture Conditions

We used NSCLC cell lines (gifts of A. Gazdar, H. Oie, and J. Minna, National Cancer Institute) NCI H596 (passage 24, adenosquamous carcinoma), NCI H226 (passage 44, squamous carcinoma), NCI H322 (passage 61, adenocarcinoma), and NCI H460a (passage 115, large cell carcinoma). All were positive for cytokeratin according to immunoperoxidase staining (Vector Laboratories, Inc., Burlingame, Calif.) and formed tumors when 10^6 cells were injected sc into *nu/nu* mice. Each cell line was cloned by adding trypsinized single-cell suspensions to confluent, irradiated (50 Gy) feeder layers of C3H10T1/2 cells as previously described (*9*). The clones were designated H226b, H322a, H460a, and H596b. Genetic signature analysis of clones demonstrated human origin, no HeLa contamination, differing genetic origins among cell types, and uniformity of genetic origin within each clone. The cell lines were adapted for growth in the serum-free defined medium ACL-3 with added growth factors (*6*). Cells

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were also grown in short-term culture in ACL-3 medium without added growth factors.

Quantitation of Epidermal-Growth-Factor-like Activity

The epidermal growth factor (EGF)-like activity released into the medium and retained by the NSCLC cell lines was assessed by a competitive binding assay of ^{125}I-labeled EGF bound to A431 cells after partial purification. Spent medium (serum-free) was obtained from nearly confluent cells in 175 cm^2 flasks after a 48-h incubation and dialyzed against 0.1 *M* acetic acid. The cells were scraped and extracted in an acid-ethanol solution (73% ethanol, 0.18 *M* HCl, 0.5 m*M* PMSF, and 2μg/mL pepstatin), followed by Dounce homogenization, and a 16-h incubation at 4 °C. The extract was centrifuged (10 000*g* for 10 min), and the supernatant was dialyzed against 0.1 *M* acetic acid, followed by lyophilization. The pellet was saved for protein determination. The samples were then dissolved in 0.1 *M* acetic acid centrifuged at 100 000*g* for 30 min, and 1 *M* Tris base was added to the supernatant to adjust the pH to 7.0–7.6. Aliquots of the samples were mixed with ^{125}I-labeled EGF and incubated for 1h at room temperature with formalin-fixed A431 cells (10^4 cells/well in 24-well plates). The specifically bound radioactivity was determined after washing and lysing the cells. EGF-like activity was measured from a standard curve, where known amounts of unlabeled EGF or TGF-α were incubated with the radioactive EGF. The amount of ^{125}I-labeled EGF added per well ranged from 0.5 to 1.5 ng and was optimized for each assay. Medium not exposed to cells contained no detectable inhibitory activity.

Growth Factor Responses

Individual growth factors were added in the following concentration ranges: EGF, 10^{-3} to 10^2 ng/mL; TGF-α, 10^{-3}–10^2 ng/mL. Cells (10^4) were added to 96-well microtiter plates in RPMI-1640 with 5% fetal bovine serum (FBS). After 18 h at 37 °C, the cells were labeled with 1 μCi/well of tritiated-thymidine ([^{3}H]Thd) for 2 h. The cultures were harvested with a PHD Cell Harvester (Cambridge Technologies, Inc.), and [^{3}H]Thd incorporation was measured with a scintillation counter. The AB-3 anti-TGF-α antibody (Oncogene Science, Manhasset, N.Y.) was added at initiation of culture in the presence (100 n*M*) or absence of TGF-α. For experiments with suramin, cells were tested after reaching confluence, and the medium was replaced with 0.2 mL/well of serum-free medium containing various concentrations of suramin or growth factors.

The effect of suramin on the growth of cells was measured on cells (2×10^3/mL) plated in triplicate in 24-well tissue culture plates in RPMI medium with 5% FBS. After overnight incubation, the medium was changed to RPMI-1640 with 1% FBS and various concentrations of suramin or growth factors. Cells were trypsinized at specified intervals and counted. These studies were done in parallel with the [^{3}H]Thd uptake studies described above.

Plasmid Construction

A 2-kb genomic DNA from the K-ras proto-oncogene was subcloned into an Apr-1-neo vector in both sense (S) and antisense (AS) orientations. A 2-kb Eco RI/Pst I fragment containing second and third exon sequences together with adjoining flanking intron sequences was isolated from the SP6 vector (Oncogene Sciences), and Klenow enzyme was used to make blunt ends. Apr-1-neo vector was digested with Bam HI, and blunt-end ligation was performed to obtain the Apr-1-neo AS or Apr-1-neo S constructs.

K-ras Antisense Construct

A 302-bp genomic DNA of K-ras gene was amplified by polymerase chain reaction (PCR) spanning the third exon with part of intron sequences. PCR-amplified DNA was subcloned into a bluescript vector. S and AS RNA probes were synthesized using either a T7 or T3 promotor. Probes were used for the detection of K-ras message in H460a cell lines expressing either S or AS K-ras RNA.

RNA PCR Analysis

cDNA synthesis was carried out in a total volume of 20 μL containing 2 μg of total RNA and oligo (dT) as a primer (*10*). A portion of the cDNA corresponding to the first and second exon segments was amplified to monitor the level of endogenous K-ras mRNA using a 5′ amplimer from first exon and a 3′ amplimer from the second exon. Amplification of cDNA was done by combining 2 μL of diluted cDNA solution with 18 μL of PCR reaction buffer. Denaturation, annealing, and extension were done at 92 °C for 1 min, 51 °C for 1 min, and 74 °C for 1 min, respectively. However, annealing temperatures for N-ras and H-ras were 44 °C and 42 °C, respectively. In addition, two amplimers were also used in the same reaction mixture to amplify a 118-bp fragment of p53 gene as a control. PCR products were either transferred onto a membrane and hybridized with ^{32}P-labelled cDNA probe or alternatively, they were directly labelled during the last cycle of amplification by adding 1μCi of ^{32}P dCTP. The labelled PCR products were loaded and electrophoresed on an 8% nondenaturing polyacrylamide gel. The gel was photographed after ethidium bromide staining, subsequently dried, and exposed to x-ray film overnight at −80 °C.

Tumor Samples and Isolation of DNA

Archival specimens comprising formalin-fixed, paraffin-embedded tissue blocks were obtained for 10 esophageal squamous cell cancers and 14 adenocarcinomas, with histologically normal esophagus from the resection margin paired with each tumor. Barrett's epithelium, with varying degrees of dysplasia, was identified in association with seven of the 14 adenocarcinomas. The remaining seven adenocarcinomas met strict criteria confirming their esophageal etiology. These included the findings (on endoscopy or radiology or at surgery) of at least 75% of the

tumor mass in the lower third of the esophagus, invasion of peri-esophageal tissues, minimal gastric involvement, and the clinical symptom of dysphagia, indicative of esophageal obstruction (*11*).

For each specimen, up to six unmounted, 5-μm serial sections were prepared. One section was stained with hematoxylin-eosin to facilitate accurate identification of tumor, normal tissue, or, where available, Barrett's epithelium, which was accurately removed using a scalpel to scrape tissues from each serial slide. This method ensured that only the tissues of interest were removed. For tumors, over 90% of cells removed from each slide appeared histologically malignant, and "contamination" with adjacent nonmalignant cells was avoided. Specimens were dewaxed by washing in xylene, and DNA was recovered according to the standard methods of Proteinase K/sodium dodecyl sulphate (SDS) digestion, phenol-chloroform extraction, and ethanol precipitation (*12,13*).

Single-Strand Conformation Polymorphism Analysis

Single-strand conformation polymorphism analysis (SSCP) (*14*) was performed directly by adding 0.5 μL of α P-32 dCTP (3000 Ci/mmol, 10 mCi/mL; ICN, Irvine, Calif.) to the PCR reaction during the last 10 cycles of amplification. A 1/10 volume of 100 m*M* EDTA/1.0% SDS was added to each completed reaction. Prior to electrophoresis, 1 μL of the solution was mixed with 1 μL of dye mix (95% formamide, 20m*M* EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol), and samples were heated to 80 °C prior to loading. Electrophoresis was performed on nondenaturing 12% polyacrylamide (stock 29.2% acrylamide, 0.8% bis-acrylamide), 10% glycerol gels, at 30 watts for up to 8 hours, using a sequencing type apparatus (S2, BRL), with 30 cm × 40 cm plates, and 0.4 mm spacers. After brief fixation with 10% methanol and 10% acetic acid, gels were dried (Biorad 583 gel dryer) for 1 h, and autoradiography was performed. Each tumor sample was electrophoresed with its corresponding normal tissue to act as an internal control. In addition, each gel included positive and negative controls (tumor with known point-mutation and normal) and a PCR-amplified, labelled cDNA to localize the bands of interest.

RESULTS AND DISCUSSION

Growth Factors and Their Receptors

Growth factor receptors and growth factor receptor-like molecules of the erbB tyrosine kinase family are expressed by NSCLC cells but not by SCLC. These genes are implicated in retrovirally induced tumors, and activation of the overexpressed normal receptor gene is sufficient for transformation of NIH 3T3 cells (*15*). The neu/erbB2 oncogene was identified in an ethylnitrosourea-induced rat neuroblastoma and is activated by a point mutation (*16,17*). In human tumors, the mechanism of activation appears to be over-expression (*18,19*). Schneider and coworkers studied erbB2 expression in NSCLC (*20,21*). Amplification of the erbB2 gene occurred in 10% of 60 paired samples of NSCLC compared with corresponding normal lung. NSCLC cell lines expressed high levels of erbB2 mRNA with adenocarcinomas having the highest levels. Surgical specimens of NSCLC showed elevated erbB2 expression in six of 16 samples compared to paired normal lung. SCLC showed minimal or no erbB2 expression. Thus, elevation of erbB2 expression may be associated with the development or progression of some NSCLC. Kern and coworkers reported expression of erbB2 in adenocarcinomas predicted reduced survival (*22*).

Cells that produce a growth factor and coordinately express its receptor may develop an autocrine growth loop in which production of the growth factor stimulates cell proliferation. Gastrin-releasing peptide is produced by SCLC cells (but not by NSCLC cells), which also express high-affinity receptors for it (*23*). NSCLC expresses the epidermal growth factor (erbB1) receptor (EGFR), which suggested to us that a ligand for this may function as an autocrine growth factor (*24*). Both TGF-α and EGF bind to the EGFR.

Previous studies indicated that medium conditioned from A549-1 lung carcinoma cells can promote the growth of human NSCLC cells in culture. This conditioned media is known to contain TGF-α and exogenous TGF-α added to cultures increased colony formation (*25*). A TGF-α/EGFR autocrine loop was investigated in vitro with cloned NSCLC cell lines by Putnam and coworkers (*26*). None of the cell lines expressed EGF by Northern analysis. All cell lines expressed TGF-α mRNA. The investigators studied the biologic response to and production of TGF-α by these cell lines. Each cell line expressed EGFR by [^{125}I]EGF competitive binding and Scatchard analysis and by phosphorylation. The receptors were functionally active as determined in immune complex kinase assays (*27*). H322a, H226b, H596b, and H460a cells showed stimulated [^{3}H]Thd uptake in culture following addition of TGF-α and EGF. Exogenously added TGF-α increased colony formation in soft agar for three of the four cell lines in media containing serum. The H596b cell line failed to form colonies with or without TGF-α. All cell lines expressed some TGF-α mRNA, although to differing degrees. Cell lysates and spent media competed for EGFR binding with EGF, thus showing production of TGF-α-like activity.

We observed differences in the mechanism of autocrine growth stimulation among the four cell lines. H322a and H226b cells were specifically growth inhibited by the anti-TGF-α monoclonal antibody AB-3 (Oncogene Sciences, Mineola, N.Y.) at low cell density, which suggests the antibody blocks an autocrine growth loop. This inhibition was dose dependent and did not occur following addition of the isotype identical monoclonal antibody MOPC21. However, the AB-3 antibody did not alter the growth of H460a and H596b cells even though these cells express EGFR and secrete TGF-α. We postulated an "internal autocrine loop" in these cells with proTGF-α binding to cytoplasmic receptor. Brachmann and coworkers solu-

bilized proTGF-α and found that it induced tyrosine autophosphorylation of EGFR in intact receptor-expressing cells and that it stimulated anchorage-independent growth of NRK fibroblasts (*28*). Thus, both proTGF-α and TGF-α can function as autocrine growth factors. Possibilities include interaction of proTGF-α with a cytoplasmic form of the EGFR as well as EGFR expressed on adjacent cells.

Suramin, which blocks binding of ligand to receptor in other autocrine systems, inhibited the growth of the cell lines not inhibited by AB-3 (*29,30*). Addition of TGF-α reversed inhibition by suramin. This appeared to be specific as the addition of PDGF did not reverse suramin-mediated inhibition. This suggests that autocrine activation for this cell line occurred in the intracellular compartment between unprocessed receptor and unsecreted ligand. TGF-α appears to be an important autocrine growth factor for NSCLC cells of both squamous and adenocarcinoma histologies.

We then studied the effects of retinoic acid on EGFR expression for NSCLC cell lines. Retinoic acid treatment caused a significant increase in receptor number with no net change in kinase activity. A time course study showed that the EGFR number increases following 16 h of retinoic acid treatment but decreases after 72 h. Pulse chase and immunoprecipitation studies indicate that the increase in EGFR number is due to increased stability of the receptor protein rather than to synthesis. This coincided with a decrease in the mRNA level for EGFR after 16 h of retinoic acid treatment. The H460a cell that showed these changes in EGFR expression also showed a reduced growth rate with the addition of retinoic acid to culture.

Ras Activation

Activation of the K-ras oncogene may lead to tumor progression in adenocarcinoma of the lung (*31–33*). It is activated in about one third of adenocarcinomas arising in patients with a history of heavy smoking. However, studies of premalignant lung lesions have not been done to determine if such mutations exist at the precancerous stage as is the case for adenocarcinoma of the colon (*34*). The presence of a K-ras mutation is associated with a poorer prognosis.

Our laboratory used an AS construct to study the effects of eliminating expression of a mutant K-ras oncogene in NSCLC cells (*35,36*). A homozygous mutation at codon 61 was detected in the NCI-H460a large cell undifferentiated NSCLC cell line clone with a normal glutamine residue (CAA) substituted by histidine (CAT) using hybridization with specific oligonucleotide probes. Direct PCR DNA sequencing confirmed this. An AS K-ras RNA construct selectively blocked the production of mutant p21 so the contribution of the mutated p21 protein to the malignant phenotype could be studied. A recombinant plasmid clone was constructed using a wild-type 2-kb K-ras genomic DNA segment carrying second and third exons with flanking intron sequences subcloned into an Apr-1-neo expression vector in AS orientation. The intron sequence used has no homology with portions of the corresponding H-ras intron where the sequence is known. We postulated that this construct would mediate specific inhibition of K-ras with preservation of H-ras and N-ras.

Previous studies with uptake of ras AS oligonucleotides by cancer cells resulted in cell death instead of regulated growth (*37*). This is probably because functioning p21 is necessary for cell viability, and the oligonucleotides unselectively blocked p21 expression. Nonselective blockade of oncogene expression can therefore be toxic to both normal and cancer cells.

An additional novel feature of this construct was the use of a beta-actin promoter that can constitutively direct synthesis of RNA in a human tumor cell. The 2-kb DNA insert was stably integrated into H460a cells as shown by Southern hybridization. Northern blot analysis detected expression of AS RNA. Western blot analysis showed 95% reduction in K-ras p21 protein synthesis in the clones expressing the AS RNA, while H460a cells and S K-ras clones showed unchanged levels of K-ras p21 protein. Total p21 detected with a pan-ras monoclonal antibody showed only a slight decrease in the AS clones, suggesting other ras genes were not affected. To confirm this, expression of ras genes was measured by cDNA PCR. The cDNA synthesized from the total RNA was subjected to PCR amplification using amplimers correspoding to the 5′-end of the first exon and the 3′-end of the second exon. Because the AS RNA was generated from the second and third exons of K-ras, PCR-amplified cDNA represented the level of endogenous K-ras mRNA. N- and H-ras specific oligonucleotides were used to determine expression of their respective genes. A 118-bp segment of endogenous p53 was coamplified in the same reaction mixture as an internal PCR control.

Cells expressing AS RNA showed complete inhibition of K-ras mRNA synthesis. There was no change in H-ras or N-ras expression in either AS or S transfectants. AS transfectants showed a three-fold reduction in growth compared to sense transfectants and parental H460a cells but continued to grow in culture. Expression of AS K-ras RNA reduced the growth rate of H460a tumors in *nu/nu* mice. Tumorigenicity of cell lines expressing AS RNA was assessed by subcutaneous injection of 10^5 cells in *nu/nu* mice. Unmodified H460a cells formed tumors in all mice in 15 days. No tumor developed in mice injected with H460a AS cells during 120 days of observation although H460a cells transfected with Apr-1-neo S plasmid formed tumors similar to H460a cells.

These experiments show that in H460a cells engineered to synthesize AS K-ras RNA, the levels of K-ras mRNA and K-ras p21 protein are dramatically reduced. Our studies show that a construct can be made that distinguishes among members of the ras family. Our data show that AS RNA generated from the genomic DNA of the K-ras gene can specifically inhibit K-ras expression. Inhibition of K-ras reduced growth rate of H460a cells but did not alter cell viability or continued growth in culture. This suggests that redundancy in p21 expression may compensate for absence of expression by one member of this family so that functions essential for maintenance of cell viability are preserved. This technique provides an opportunity to

determine the effects of selective inhibition of oncogenic protein expression on the malignant phenotype.

The p53 Gene

The p53 gene encodes a 375-amino acid phosphoprotein that complexes with host proteins such as large T-antigen and E1B (*38*). Missense mutations are common for the p53 gene and are essential for the transforming ability of the oncogene. The mechanism of p53 transformation is controversial. The wild type p53 gene may directly suppress or indirectly activate genes that suppress uncontrolled cell growth. Thus, absence of the wild type p53 or inactivation of wild type p53 may contribute to transformation. However, some studies indicate that the presence of the mutant p53 may be necessary for full expression of the transforming potential of the gene. Mutations of p53 are common in a wide spectrum of tumors. These mutations occur in both NSCLC and SCLC cell lines and in fresh tumors (*39, 40*). The precise role of these mutations in oncogenesis and the mechanisms involved are subjects of active investigation.

Recent data suggests that mutations in the p53 gene may precede the onset of clinical malignancy. Malkin and coworkers reported mutations in the p53 gene in lymphocytes and noncancerous fibroblasts of individuals from families with the Li-Fraumeni syndrome (*41*). Some family members have the germline mutation but have not developed clinical evidence of cancer.

Casson and coworkers identified p53 mutations in cancer of the esophagus (*42*). Mutations were identified in both adenocarcinoma and squamous carcinoma of the esophagus using SSCP analysis for screening and direct sequencing of putative mutations. This prompted study of columnar epithelial cells lining the esophagus (Barrett's esophagus), which is a premalignant lesion frequently associated with the development of adenocarcinoma of the esophagus. Studies of cells derived from Barrett's esophagus showed p53 mutations in four of seven specimens from patients with associated adenocarcinoma (*42*). Mutations in the p53 gene were not present in corresponding normal esophageal mucosa indicating that the mutations were somatic. This is the first demonstration of a p53 mutation in a premalignant lesion, and it suggests that p53 may contribute to the development of malignant changes in preneoplastic lesions.

Mutations in the p53 gene are common in lung cancer, and therefore studies of the mechanism of p53 are important for understanding its contribution to the development of these cancers (*39,40,43*). Mukhopadhyay and coworkers transfected NSCLC cell lines with plasmids containing wild type p53 cDNA in either S or AS orientation (*44*). Cell lines with differences in p53 expression were used. The H322a lung adenocarcinoma cell line expresses the mutant p53 protein as shown by the presence of high levels of endogenous p53 mRNA and phosphorylated protein. We showed the H322a cell line has a G:T transversion at codon 248 (Arg to Leu) with absence of the wild type allele. The H226b cell line is homozygous for the wild type p53. Expression vectors for S-p53 and AS-p53 cDNA with a beta-actin promoter were constructed to study the effect of wild type p53 expressed in lung cancer cells with mutant p53 and the effects of reducing wild type and mutant p53 expression.

Stable transfectants of p53 mutant cells expressing S-p53 could not be rescued (Table 1). Failure to isolate colonies expressing S-p53 RNA in cells with homozygous mutant alleles shows that wild type p53 can suppress transformation in lung cancer cells expressing a mutant p53.

In general, transfection with AS-p53 reduced colony formation by cells with endogenous mutant p53. This indicates expression of mutant p53 contributes to the transformed phenotype. Two H322a (mutant endogenous p53) isolates transfected with AS-p53 (one clone C4 shown in Table 1) expressed very low levels of p53 protein. Two H226b (wild type endogenous p53) isolates transfected with AS-p53 (one clone C5 shown in Table 1) also showed decreased p53 expression. These clones showed a marked increase in growth rate and tumorigenicity in *nu/nu* mice. This suggests that in some instances the mutant p53 gene continues to express growth control functions. In this case, elimination of residual mutant p53 function enhanced tumorigenicity. As expected, reduction in wild type p53 expression contributes to increased tumorigenicity.

Our studies show that for lung cancer cells wild type p53 is dominant and can suppress the malignant phenotype in cells with mutant p53. The presence of the mutant p53 confers gain of function, but in some cases reduction in mutant p53 expression increases tumorigenicity.

These data suggest that growth factors and dominant and suppressor oncogenes play important roles in the maintenance of the transformed phenotype for NSCLC cells. The characteristics of four human NSCLC cell lines that we have studied are summarized in Table 2. These cell lines are representative of all the major NSCLC histologies. All cell lines showed growth factor receptor expression and growth stimulation by a TGF-α autocrine loop. For three cell lines either a p53 or K-ras oncogene mutation was present. Reversal of a single abnormality has profound effects on the transformed phenotype for indi-

Table 1. Characteristics of NSCLC cell lines transfected with the p53 cDNA*

Cell line	H322a	H322a	H226b
Clone	ND†	C4	C5
Endogenous p53	Mutant	Mutant	Wild type
Transfected construct	S	AS	AS
Colonies formed	0	+	+
p53 expression‡	ND	D	D
Tumorigenicity	ND	+ +	+ +

*Cells were transfected by electroporation with wild type p53 cDNA in the sense (S) or antisense (AS) orientation in the Apr-1-neo plasmid (*36*). Expression of p53 protein was measured by immunoperoxidase staining with PAb 1801 monoclonal antibody. Tumorigenicity was measured by the growth rate of tumors from 10^6 cells injected subcutaneously in *nu/nu* mice.

†ND = not done

‡Compared to untransfected cells; D = decreased expression.

Table 2. Summary of relative growth factor receptor expression, autocrine growth stimulation by TGF-α, and oncogene mutations in four NSCLC cell lines*

	Squamous H226b	Adenocarcinoma H322a	Large cell H460a	Adenosquamous H596b
EGFR	3+	2	1+	3+
erbB2	1+	2+	1+	2+
TGF-α autocrine loop	Yes	Yes	Yes	Yes
Ras mutation	No	No	Yes	No
p53 mutation	No	Yes	No	Yes

*EGFR expression was measured by Scatchard analysis. Expression of erbB2 was measured by mRNA dot blots.

vidual cell lines. It is possible that only one or a few genes need to be targeted to achieve a therapeutic effect. Future studies will need to focus on both identification of other genetic events critical for cancer cell development and progression and on delivery systems for agents that reverse these events.

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The ras Gene Family in Human Non-Small-Cell Lung Cancer[1]

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ABSTRACT—The three ras genes code for proteins with a putative role in cellular signal transduction. They belong to a larger family of small guanosine-triphosphate (GTP)-binding proteins. The ras proteins acquire transforming activity when amino acids are substituted at one of a few specific sites, as a result of a point mutation in the gene. In about one third of adenocarcinomas of the lung, a K-ras mutation is present in codon 12 of the gene. Patients with early stages of K-ras mutation-positive tumors have a very unfavorable prognosis, even if apparently radical resection of the tumor has taken place. K-ras mutations are very rare among nonsmokers, and it is reasonable to assume that carcinogens in tobacco smoke directly cause the mutation. The types of ras mutations found in lung cancer are different from those in gastrointestinal malignancies. Colon cancer is mainly associated with mutations leading to substitution of the normal glycine at amino acid position 12 of K-ras by either valine or aspartic acid, and mutations in N-ras are not exceptional. In contrast, the predominant mutation in lung cancer leads to substitution of cysteine in codon 12. Several other members of the ras gene superfamily are also expressed in human lung cancer, but a possible relationship with lung tumorigenesis remains to be established. [J Natl Cancer Inst Monogr 13:23–29, 1992]

Among the oncogenes known to be associated with human malignancies, the ras genes occupy a position of special prominence. Few other genes rival the ability of ras to induce malignant transformation in vitro, and no other oncogene has been encountered in an activated state in such a wide variety of tumor types. In particular, activated ras genes have been demonstrated in a range of common tumors, such as colon cancer, pancreatic cancer, and lung cancer, raising hope that the deepened insight into their pathogenesis might eventually benefit the large number of patients afflicted with these disorders.

The ras genes have also provided the first direct link between the molecular genetics of cancer and chemical carcinogenesis (*1*). Activation of ras genes by point mutation can occur as a direct consequence of exposure to carcinogens in several animal models (*2*). In some cases, the specific type of ras mutation (the base substitution and its position in the ras gene) can be traced to the specific chemical alteration induced by the carcinogen (*3*).

Perhaps the most distinguishing characteristic of ras oncogenes, however, may be the fact that the presence of the activated oncogene can be determined not only conveniently but also unambiguously in human tumor tissues. Because the activating mechanism consists of a point mutation, the activating alteration in the gene is either present or absent, and no confusion can arise concerning possibly "high-but-normal" versus inappropriately high expression levels, as is the case with many (onco)genes thought to be activated by defects in expression regulation.

THE ras GENES

The three well-characterized ras genes, H-ras, K-ras, and N-ras belong to a superfamily of genes coding for small monomeric guanosine triphosphate (GTP)-binding proteins [for review see (*4*)]. The three ras genes code for highly homologous 21kd proteins ($p21^{ras}$) that are localized at the inner side of the cell membrane. Their homology with G proteins strongly suggests a function in signal transduction (*3*). The ras proteins can exist in two states: 1) an active state in which guanosine triphosphate is bound to the molecule and in which presumably a signal is relayed into an as yet unidentified secondary messenger pathway and 2) an inactive state in which the GTP has been hydrolyzed to guanosine diphosphate (GDP) (*3*). The ras proteins possess intrinsic GTPase activity, which eventually leads to their inactivation, but this inactivation is greatly enhanced by a second protein, called the GTPase activating protein (GAP) (*5*). GAP has been shown to bind to the domain that is involved in the transduction of the ras signal, the "effector" domain of $p21^{ras}$ (*6*). It is currently unclear whether GAP is involved only in enhancing ras GTPase activity or whether it is also the effector molecule for $p21^{ras}$ (*7, 8*). The discovery that GAP may be phosphorylated by the activated platelet-derived growth factor (PDGF) receptor has provided evidence for a direct biochemical link between tyrosine kinases and the ras signaling pathway (*9, 10*).

The ras genes have been highly conserved in evolution, and expression of one or more of the three genes can be demonstrated in most of even all mammalian cells (*11*). Thus, the presence of ras proteins must be essential for the

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normal physiology of the cell. The event that turns a normal ras gene into an oncogene is a single point mutation that leads to a single amino acid substitution in the encoded protein (*3*). Only point mutations resulting in loss of the intrinsic GTPase activity (thus causing inability of $p21^{ras}$ to switch back to the inactive state) appear to be associated with transforming activity of the protein. Thus, activating mutations are invariably found in the GTP-binding regions of $p21^{ras}$ (*12*). In practice, virtually all relevant mutations are found in codons 12, 13, or 61 of H-, K-, and N-ras (*13*).

THE DETECTION OF ACTIVATED ras GENES IN HUMAN TUMORS

The standard method to detect the presence of an activated ras gene in a given DNA sample was the NIH/3T3 focus assay (*14*), until detection with mutation-specific oligonucleotides was developed by Bos's group (*15*). Because the activating point mutations of the ras genes are essentially confined to only three codons of the gene, only a limited number of oligonucleotides have to be synthesized, each detecting one of the possible point mutations within the gene. Point mutations can then be found by carefully washing the hybridized DNA gels or filters at increasing temperatures, in such a way that only fully matched probes are retained (*16*). This oligonucleotide assay proved to be highly specific and reliable but required microgram amounts of undegraded DNA. The later addition of in vitro amplification of the DNA sequences of interest, employing the polymerase chain reaction (PCR) (*17*), has eliminated these restrictions. Even the minute quantities of DNA fragments that can be isolated from single sections of formalin-fixed, paraffin-embedded tumor samples in pathology archives now allow the oligonucleotide hybridization assay to be performed with success (*18*). Mixing experiments have shown that the oligonucleotide hybridization assay is sufficiently sensitive to reliably identify tumor populations that make up only 5% of all cells in the sample (*19*).

These sensitive and specific techniques to detect point mutations in ras oncogenes have been used to screen various series of human tumors (*13*). It is now clear that an overall 10%–15% of all human cancer specimens contain such a mutation. H-ras point mutations have been reported in a low percentage of thyroid and bladder cancers, and N-ras mutations have mainly, but not exclusively, been associated with malignancies of the hematopoietic system (*13*). For unexplained reasons, K-ras mutations are preferentially found in adenocarcinomas, albeit with frequencies that depend on the origin of the tumor: 80%–90% in pancreatic cancer (*20,21*), 50% in colorectal cancer (*22,23*), and 30% in adenocarcinoma of the lung (*24,25*) (*see* the following section on lung cancer) but virtually never in adenocarcinoma of the breast or of the ovary (*26,27*). Gene amplification or overexpression of ras proteins has also been reported, but the former is quite rare (*28*), and the significance of overexpression is difficult to assess because of concerns about the specificity of some of the monoclonal antibodies used (*29*) and because of the problem of distinguishing between high expression and overexpression (*30*).

ras ONCOGENES AND LUNG CANCER

The first indications that ras mutations might be involved in the pathogenesis of human lung cancer came from human lung cancer cells lines (*31,32*) and from anecdotal reports of ras mutations in tumor biopsies of patients (*33*). Prompted by these findings, we investigated a series of 39 primary non–small-cell lung cancer specimens, obtained at thoracotomy for potentially resectable disease (*34*). Five of the 10 adenocarcinomas, but none of the other subtypes, contained a K-ras mutation ($P < .001$, chi-square test). Since that time we have expanded our series considerably (Table 1) (*24*), and the specificity of K-ras for adenocarcinomas remains striking though not absolute. Even with complete specificity, a few mutations in tumors with undifferentiated histology might be expected because of the indistinct delineation of this subtype from adenocarcinoma, but rare occurrences of K-ras gene mutations in epidermoid carcinomas of the lung have been documented (*33*). The now classic experiments of Barbacid and coworkers, in which rats developed tumors only weeks after a single injection of a carcinogen, suggest that the type of ras mutation found in the tumor may depend on the nature of the carcinogen (*35,36*). This principle may explain the differences in ras mutations between colonic tumors and lung adenocarcinomas. In our own lung adenocarcinoma series, over 80% of the mutations in

Table 1. Distribution of ras point mutations in human lung cancer according to histologic classification

	K-ras codon 12	K-ras codon 61 (K61), N-, or H-ras codon 12 or 61 (N12, N61, H12, or H61)
Small-cell lung cancer	0/6*	0/6
Non–small-cell lung cancer		
Epidermoid carcinoma	0/34	0/34
Adenocarcinoma	27/103	1/51 (H12)
Large-cell carcinoma	1/23	2/23 (K61 and N61)
Carcinoid	0/15	ND†

* Numbers positive versus total samples investigated.
† ND = Not determined.

K-ras codon 12 are guanine-to-thymine transversions, and we found a different spectrum of K-ras codon 12 point mutations in a series consisting of primary and metastatic colon adenocarcinoma samples (Table 2) (*37*). In a recent Japanese study, a similar observation was made: 8 out of 10 point mutations in codon 12 of K-ras in lung adenocarcinomas were guanine-to-thymine transversions (*38*). Also in the p53 gene, a gene that is frequently altered in human lung tumors, guanine to thymine transversions are common point mutations (*39*).

DIFFERENCES BETWEEN K-ras-POSITIVE AND K-ras-NEGATIVE TUMORS

Even in lung adenocarcinomas, only a minority of about 30% contains a mutationally activated K-ras oncogene. In theory, activation of K-ras might in other cases result from amplification of the nonmutated gene or from overexpression. We have, however, found no evidence of K-ras amplification in 44 lung adenocarcinomas (*28*), and others have found only one case of ras oncogene amplification in 35 lung tumor samples (*40*). These results indicate that gene amplification is not an important alternative activating mechanism in these tumors. Using semiquantitative PCR, we have also determined K-ras mRNA expression levels in a series of 24 non-small-cell lung cancer specimens (*41*). Most tumors expressed K-ras at a somewhat higher level than normal lung tissue cells, but not at levels similar to those known to have transforming activity in vitro, and there were no apparent differences in expression between adenocarcinomas and other subtypes. Thus, the acquisition of a point mutation is quite likely the only important mechanism of K-ras activation in lung cancer.

It must be kept in mind, however, that the 30% frequency of K-ras activation in adenocarcinoma of the lung is derived from studies of patients who had (clinically) early disease and who were consequently subjected to attempts to resect their tumor, as in the case of colorectal cancer. It could well be that K-ras mutations are more frequent in advanced disease, in analogy to the case of colorectal cancer (*42,43*). This question is being addressed in ongoing studies. Even if this were true, most lung adenocarcinoma patients do not have a K-ras mutation in their tumor, and consequently, a K-ras mutation is not an obligatory step in the pathogenesis of this tumor. This conclusion allows two possible (although not necessarily mutually exclusive) hypotheses: Either 1) K-ras mutations constitute a step in malignant transformation, but one that can be substituted for by another (unknown) event, or 2) a K-ras mutation renders a selective growth advantage to a malignant cell acquiring the mutation prior to, during, or after transformation.

If the latter of the two hypotheses were valid, one would expect an adenocarcinoma with a K-ras mutation to be biologically (and thus clinically) different from a tumor without such a mutation. Thus, we examined additional adenocarcinoma patients and retrospectively compared the clinical characteristics between the K-ras-positive and K-ras-negative groups. In our first explorative study, the differences appeared to be minor (*24*). Patients with K-ras mutations had tumors in somewhat earlier stages, but the significance levels in this rather small study were only borderline. The inclusion of additional patient samples together with the now available survival data did suggest that patients with a K-ras mutation have biologically more aggressive tumors. We studied 69 patients whose lung adenocarcinomas had been completely resected and who were believed to be free of tumor after operation. Forty-eight patients had stage I disease, 14 had stage II, and 7 had stage IIIa (*25*). Nineteen tumors contained a K-ras point mutation. The K-ras point-mutation-positive group did significantly worse than the K-ras-negative one, with poorer disease-free interval and overall survival (log-rank tests: $P = .038$ and $P = .002$, respectively). This difference was readily detectable despite the fact that the stages of disease of the K-ras positive group were actually slightly more favorable than in the K-ras negative group.

A second difference between patients with and without K-ras mutations in their lung adenocarcinomas concerns their smoking histories. We have speculated, based on borderline statistical significance in a retrospective analysis of a small number of patients, that K-ras mutations may be directly caused by exposure to tobacco smoke (*24*). In a collaborative study with Drs R.H. Hruban and G.J.A. Offerhaus of The Johns Hopkins University Hospital in Baltimore, we prospectively studied adenocarcinomas of 27 patients who were smokers and of 27 matched patients who had never smoked (*44*). K-ras mutations were found in two nonsmokers and in eight smokers ($P = .044$, Fisher's exact test). These data, combined with our earlier

Table 2. Distribution of point mutations in K-ras codon 12 in lung cancer and colon cancer

K-ras codon 12	Lung adenocarcinoma	Colon adenocarcinoma
"wild-type" gly (GGT)	76	63
cys (TGT)	17	1
ser (AGT)	0	0
arg (CGT)	0	2
val (GTT)	5	11
asp (GAT)	4	15
ala (GCT)	1	1
Total mutated	27	30

findings and with additional unpublished data from our laboratory, indicate that smoking and K-ras mutations are indeed linked.

ras-RELATED GENES AND LUNG CANCER

As already mentioned, the ras proteins belong to a sizable superfamily of small monomeric GTP-binding proteins. This superfamily is characterized by the ability to bind guanine nucleotides and is structurally and functionally related to the family of G proteins. The G proteins have been intensively studied and have been shown to have a key role in signal transduction. It is of considerable interest that point mutations in G-protein genes, similar to those found in ras genes, have recently been reported in certain endocrine tumors (*45*). Over 30 small monomeric GTP-binding proteins have been described at present, in species ranging from yeast to human beings (*4*). In humans, 25 different ras and ras-related genes have been identified and (partly) characterized. All these ras-related proteins are highly conserved in the GTP/GDP-binding regions and in the "effector" domain. Eight ras-related proteins are close relatives of the three ras oncogenes, with about 50% similarity at the protein level: ralA and B (*46*); rap1A (Krev), 1B, 2A, and 2B (*47,48,49*); R-ras (*50*); and rasab (TC21) (*51*). Other relatives of ras, with about 30% similarity at the protein level, are rhoA, B, and C (*52,53*); racA and B (*54*); rasac (TC4) and rasad (TC10) (*51*); and rab1, 2, 3A, 3B, 4, 5, and 6 (*55*). The relative distances between the ras-related proteins, based on their percentage similarity in the amino acid sequences, are shown in Fig. 1. This amino acid sequence alignment illustrates the similarities between the ras-related genes but may not be identical with a true phylogenetic tree. Based on Fig. 1, three major branches of ras-related genes can be distinguished: 1) the ras, ral, and rap genes, of which both ras and rap1A influence the transformed state of NIH/3T3 fibroblasts; 2) the rab genes, which are important in vesicle transport within the cell (*4*); 3) the rho genes, which are presumably involved in the organization of the cytoskeleton and are, together with the rac genes, the only known monomeric GTP-binding proteins that are substrates for mono-ADP-ribosylation by bacterial exoenzymes (*56*). One of these genes, rap1A (Krev), is of special interest because it is able to revert v-K-ras transformed NIH/3T3 cells and is thus a candidate tumor-suppressor gene (*57*).

Because at least two families of GTP-binding proteins have now been associated with human malignancies, we have begun to explore their possible involvement in lung cancer. If we assume that amino acid substitutions in the GTP-binding domains are the most likely mechanisms of activation, screening for point mutations at the DNA level appears to be a rational strategy. To detect expression of ras-related genes we used several different mixtures of oligonucleotides directed at the highly homologous GTP-binding regions around codons 12 and 61 in the ras oncogenes to amplify all ras-related cDNAs in a single

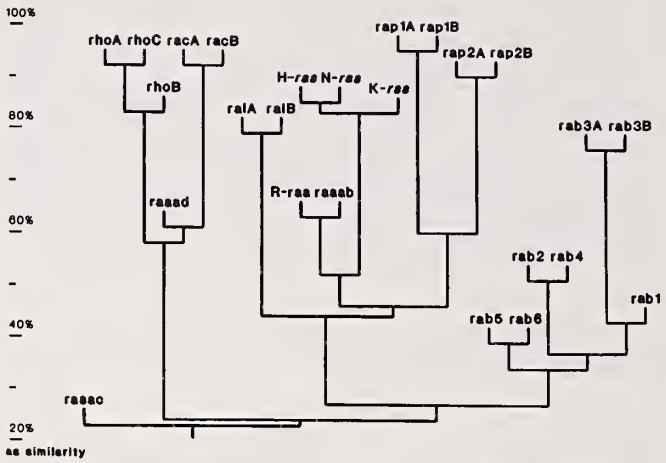

Fig 1. Grouping of the members of the ras gene family, based on similarity in amino acid sequence. DNA sequences from the 25 human ras-related genes were extracted from the Genbank and EMBL databases and translated to their respective amino acid sequences using the Genetic Computer Group package for the VAX (*64*). The resulting sequences were aligned employing the method as described by Wilbur and Lipman (*65*), using the CLUSTAL program under VMS (*66*). The ras gene superfamily can be roughly divided into three separate major branches (see text).

PCR. In these oligonucleotide mixtures, each possible DNA sequence at codon 12 or 61 from each ras-related gene is represented. The resulting PCR fragments were then slot-blotted on different membranes and hybridized with oligonucleotides specific for each of the known ras-related genes. A total of 16 primary lung tumors and five lung tumor cell lines were investigated for expression of 17 ras-related genes (rab genes were not included in this survey). Signals were obtained with the probes for K-ras, N-ras, rasac, and racA (Fig. 2), indicating that these genes had the highest levels of expression in the investigated lung carcinomas, and the remaining ras-related genes had undetectable expression levels with this method.

Because of its ability to reverse the transforming activity of ras, expression of rap1A was determined by PCR on cDNAs using rap1A specific oligonucleotides. Expression of this gene was detected in almost all specimens with this very sensitive approach (*58*). Preliminary data indicate that the rap1A gene does not harbor point mutations in human non-small-cell lung carcinomas. Further study, including a search for point mutations in the GTPase-binding domains of these genes, is needed to determine whether or not some of the ras-related genes play a part in human lung cancer.

CONCLUSIONS

Our studies have shown that mutational activation of the K-ras oncogene is clearly a pathogenetic factor in adenocarcinoma of the lung. But the mutation is present in only 30% of the tumors and is obviously not an absolute requirement for malignant transformation. Most evi-

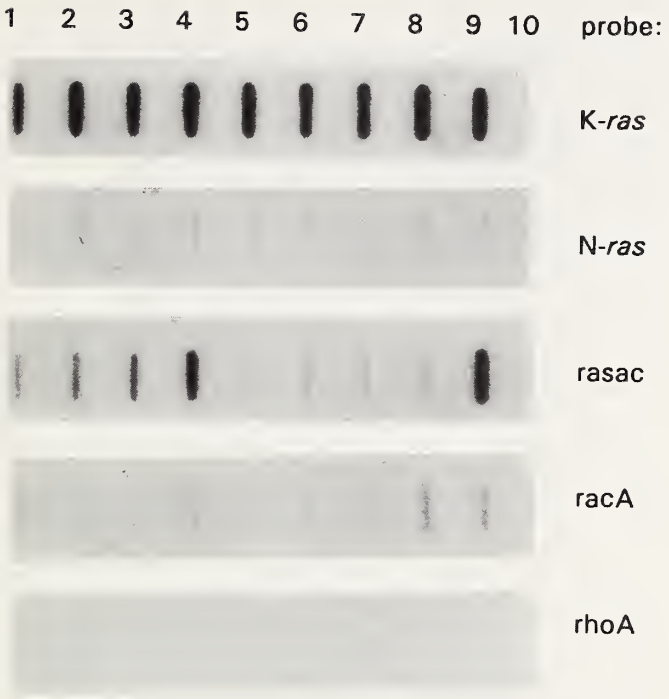

Fig 2. Detection of expression of ras-related genes in human lung carcinomas. cDNAs isolated from eight lung tumor specimens were amplified using degenerate oligonucleotides representing the most commonly found nucleotide sequences around codons 12 and 61 of ras, slot-blotted on separate membranes, and hybridized to [^{32}P]-labeled oligonucleotides specific for each of the ras-related genes. Samples 1–4, adenocarcinomas; samples 5 and 6, large cell carcinomas; sample 7, epidermoid carcinoma; samples 8 and 9, adenocarcinoma cell lines (NCI-H23 and A549, respectively); sample 10, no cDNA added. Shown are hybridizations with oligonucleotides specific for K-ras, N-ras, rasac, racA, and rhoA.

dence now points to a role for K-ras in the progression of an established tumor toward a more malignant phenotype, although the specific consequences of the activated oncogene are still poorly defined. In addition, it should be recognized that it is probably not because of their prominent role in the pathogenesis of lung cancer that K-ras mutations were among the first molecular genetic alterations recognized in this tumor. The technology to detect ras activations merely happened to mature earlier than that for other oncogenes, and the apparent prominence of the ras genes may well result from "selective perception." Tumorigenesis in humans is a process thought to require at least five separate steps, and it is likely that multiple pathways to malignancy exist for a given tumor type. Thus, ras point mutations are only a small part of the story that undoubtedly involves other oncogenes and tumor suppressor genes, already known or still elusive.

At present, the main objective of oncogene analysis in human tumors is to refine the classification of malignant disease. It appears reasonable to assume that a direct evaluation at the molecular level of parameters associated with the malignant state would allow improved prediction of clinical behavior or response to treatment. In selected clinical situations, K-ras oncogene analysis can already help in differential diagnosis (*59*). The identification of K-ras point mutations as prognostically very unfavorable in lung adenocarcinoma patients who were operated on successfully raises the question whether adjuvant therapy should be given in radically operated patients with K-ras mutations. Modern triple-drug chemotherapy regimens approach 50% response rates in suitably selected patients (*60*) and could thus be regarded as potentially beneficial in patients with only microscopic disease.

The question that must be answered before adjuvant chemotherapy is considered, is whether the K-ras mutations are associated with clinical drug resistance in humans. This possibility has been raised by a number of studies reporting diminished sensitivity of cell lines to both irradiation and cytotoxic agents after transfection of an activated ras gene (*61,62*). We are now investigating this question by treating metastasized lung adenocarcinoma patients with ifosfamide/mesna, carboplatin, and etoposide [MICE (*63*)], which will allow us to study the possible significance of K-ras mutations for drug resistance prospectively. The outcome of this study may also contribute to improved patient selection for chemotherapy in advanced disease.

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Early Events in the Neoplastic Transformation of Respiratory Epithelium

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ABSTRACT—The complex process of epithelial carcinogenesis is composed of discrete biologic events including the early activation events of "initiation" and "promotion." For lung cancer, these events are only now being elucidated. Despite the identification of possible target genes and their mutations, the "initiation" events for lung cancer remain poorly understood. The identification of these "initiation" events is a crucial step toward the development of practical molecular markers for early detection of this disease. The reversible process of tumor promotion remains somewhat enigmatic but is a promising target for chemoprevention. A wide range of substances, including asbestos and various substances in cigarette smoke, behave as tumor promoters for lung cancer. They appear to promote tumor formation by inducing cellular proliferation mediated in part by growth factors. The intracellular signals these factors provide are ultimately translated into cellular growth via steps involving nuclear transcription factors. Early response genes such as the jun and fos gene family members encode such nuclear transcription factors which are expressed in lung cancer cells and primary bronchial epithelial cells. The expression of these transcription factors is highly responsive to stimulation by growth factors including serum, transforming growth factor, and gastrin-releasing peptide. A more thorough understanding of this process will allow the development of molecular and/or pharmocologic antagonists that can interfer with the biologic process of tumor promotion and therefore function as chemoprevention agents. [J Natl Cancer Inst Monogr 13:31–37, 1992]

MULTISTAGE NATURE OF THE MALIGNANT PROCESS

The mystery of the etiology of human cancer stems in part from its enormous complexity. Pathologists have noted for years the impressive diversity of human tumors both in tissue of origin and histologic features. Even within a specific organ system, a wide spectrum of pathologies exists reflecting different cell morphologies, mitotic activity, nuclear atypia, and, ultimately, clinical course. An understanding of the process that gives rise to such diversity began when early biologic studies suggested that complex interactions between various chemical or biologic agents and their cellular targets over a period of time could induce tumors in animals. Historically, most of the initial work involved exploring the development of skin tumors in animal models. Studies by Berenblum and Shubik in 1947 (*1*) showed that when small amounts of a carcinogenic polycyclic hydrocarbon are applied to the skin of a mouse, small numbers of tumors arise, but if the treatment is followed by a noncarcinogenic phorbol ester, a much larger number of tumors develops.

These classic experiments suggested that the early biologic processes involved in the neoplastic transformation could be divided into discrete events termed initiation and promotion and the substances producing them into tumor initiators and tumor promoters. Further work in these systems determined the specific biologic characteristics that distinguish promoters from initiators. Initiators are substances that produce irreversible genetic events inherited by a whole clone of cells. Promoters, on the other hand, produce reversible effects on the cells exposed to them without transmitting those effects to other cells.

Modern molecular biology has provided mechanistic support for the multistage hypothesis of cancer development originally derived primarily from biologic observations of the neoplastic process (*2*) (Fig. 1). These molecular studies have demonstrated that the process of tumor formation involves the accumulation of multiple genetic events over a period of time, which ultimately conspire to produce malignancy. Accumulating evidence from molecular biological and cytogenetic studies demonstrates that most epithelial neoplasms contain multiple chromosomal deletions and rearrangements. In fact, although there appear to be some unique features among specific tumor types, such as lung, colon, breast, and skin, the process of malignant transformation is quite similar among most epithelial tumors. Reduction to homozygosity of loci mapping to chromosome 17 has been reported for lung, breast, and colon cancers, while chromosome 3p deletions are associated with lung and renal cancers, and chromosome 18 abnormalities are associated with colon cancer (*3–6*).

Molecular analysis of tumors from animal models provided the first clues to the chronologic sequence and biochemical nature of these genetic events. In the 7,12-dimethylbenz[*a*]anthracene (DMBA)-initiated skin tumors of mice, the target gene was identified as the ras oncogene, which undergoes a specific activating mutation. In fact, activating mutations (carcinogen specific) of this gene

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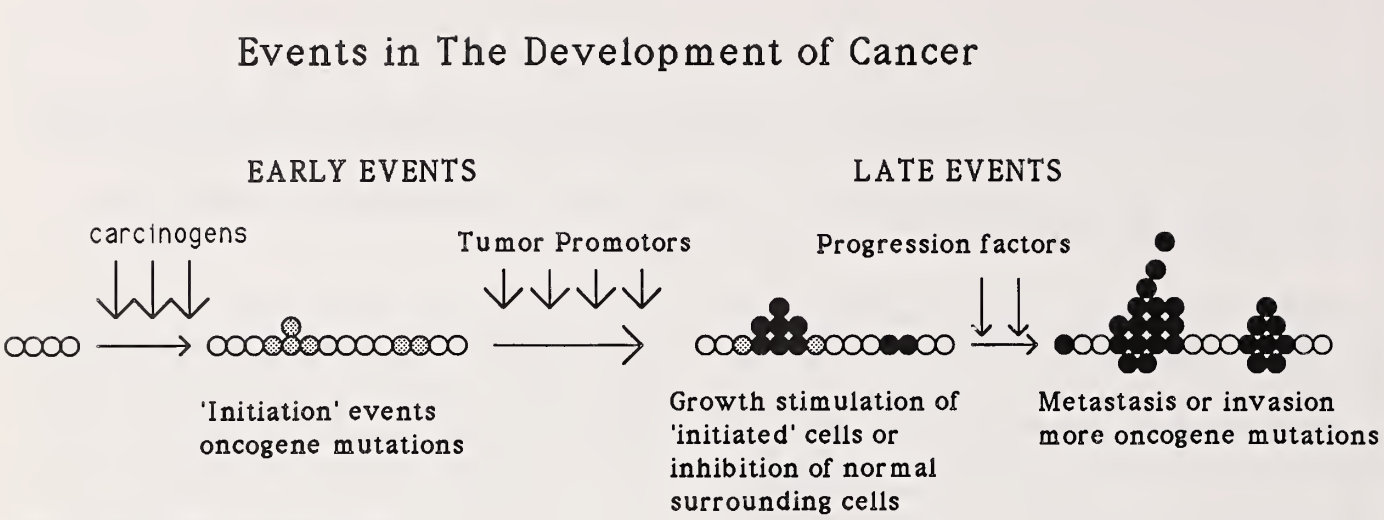

Fig. 1. Events in the development of cancer.

have been identified in several other carcinogenic model systems, suggesting that it is a frequent target gene involved in the initiation process (*7,8*). Recent work in human tumors suggests that mutations of this gene may be important as an early event during cancer development (*9, 10*). For instance, ras mutations have been found in many colonic carcinomas; additionally, in certain cases, neighboring premalignant adenomatous polyps have been found to have identical ras mutations (*10*). This suggests that activation of the ras oncogene may have been an early event in the development of these tumors. The presence of activated ras may allow the epithelial cell to have a limited proliferative advantage (under the influence of tumor promoters) over its surrounding epithelium, and thus, after clonal expansion, this clone accumulates other independent mutational events that convert it to full malignancy. Molecular analysis of a wide range of epithelial cancers has identified other genes that are frequent targets for these mutational events. They include dominant oncogenes such as c-myc, N-myc, L-myc, and Her-2/neu; the tyrosine kinases; and recessive oncogenes such as Rb and p53 (*2*).

Cytogenetic and molecular analysis of lung cancer cell lines and tumor specimens reveals an extremely complex pattern of mutation in a wide range of target genes (*11*). Among these genes are many of the ones listed above, such as ras and myc, which undergo mutational activation or deregulated expression, and the recessive oncogenes, such as p53 and Rb, which are inactivated by point mutation or deletion (*12*). Unfortunately, while these genetic lesions have been well catalogued and characterized, there is less information on their chronologic contribution to the development of lung cancer. Many of these genetic events serve as "progression events" and contribute to the invasiveness and metastatic phenotype of malignant cells but are not necessarily involved in the establishment of the neoplastic process per se as would be expected for an initiation event.

TUMOR PROMOTION

Tumor promotion has been a more diverse, ill-defined, and poorly understood entity in the malignant process. Historically, it has been best characterized in mouse model systems examining skin tumor formation, where the most frequent promotional agents used are phorbol esters, such as 12-O-tetradecanoyl phorbol-13-acetate (TPA). This system has elucidated the biologic characteristics of tumor promoters. In contrast to carcinogenic effects, TPA-induced effects are not cumulative. In addition, the dose and timing of its administration are critical. Increasing the dosing interval while still delivering the same total dose of TPA was found to decrease the tumor yield (*13*). In addition, treatment with TPA prior to carcinogenic treatment is ineffective in producing augmented tumor formation (*14*). This suggests that TPA effects are reversible and dependent on the continual presence of the agent. Finally, the dose-response curve for TPA is sigmoidal as compared to the linear curve seen with most carcinogens, suggesting that TPA interacts with a different cellular target than do initiators (*15*).

The list of agents that have been identified as tumor promoters is long (Table 1). These agents are extremely varied in terms of their chemical structure and biologic nature. In addition, they demonstrate remarkable tissue specificity. Most of these agents have been identified in animal models, but recently others have been discovered through the use of in vitro assays.

The biologic actions of tumor promoters seem to fall into several distinct types. The majority are agents that ultimately stimulate cell proliferation. For instance, TPA induces a hyperplastic skin response in the mouse skin model, and estrogens (which promote DMBA-initiated cells to form tumors) stimulate mammary epithelial cells to grow (*16*). These promotional agents presumably provide a growth advantage to an initiated cell so that it is able to outgrow the surrounding epithelium. On the other

Table 1. Tumor promoters identified for epithelial cancers

Tissue	Agent/Species
Lung	TPA/human
	Asbestos/rat
	Butylated hydroxytoluene/mouse
Skin	TPA/mouse
	TCDD/mouse
	Cigarette smoke/mouse
	Wounding/mouse
Breast	Prolactin/rat
Colon	Bile acids/rat

hand, it has also been proposed that tumor promoters function by inducing differentiation or inhibiting the growth of the surrounding normal cells (*17*). Initiated cells would lose the necessary sensitivity to these effects and therefore continue to grow. In a hepatocellular carcinoma model system, tumors can be readily induced after exposure to a wide range of carcinogens, all of which are genotoxic to normal liver cells (*18*). In addition, it has been suggested that TPA acts as a promoter in skin tumor model systems by selectively differentiating normal skin cells, thus enriching for the nonresponsive initiated cells (*19*).

In lung cancer, little is known about the identity or role of specific tumor promoters. Model systems have provided some information and several candidate agents have been identified and characterized. In the mouse lung model, butylated hydroxytoluene has been shown to significantly enhance urethane-induced tumor formation (*20*). In addition, in a heterotopic rat tracheal graft assay, both TPA and asbestos have been shown to enhance tumor yield (*21,22*). More recent work has focused on in vitro systems using clonal assays to examine the effects of a limited number of suspected promoters on cell proliferation. TPA has been shown to inhibit the growth of normal human bronchial epithelial cells (*17*). This result was reproducible and impressive in that extremely small quantities of TPA (1 to 10 ng/mL) were able to inhibit colony formation. Interestingly, there was great heterogeneity among specimens, suggesting some baseline variability in sensitivity to phorbol esters. If, indeed, tumor promotion is mediated in part through cellular proliferation, then potentially any or all growth factors active for respiratory epithelium may function as tumor promoters. Studies on bronchial epithelial cells and lung cancer cell lines have identified multiple factors that stimulate cell growth. Gastrin-releasing peptide, insulin-like growth factor 1, transferrin, and selenium have all been shown to stimulate the growth of small-cell lung cancer cell lines and/or primary bronchial epithelial cells (*23,24*).

A panoply of substances exists in cigarette smoke, and many of these substances, including nicotine and tar, appear to function as tumor promoters in in vitro assays (*25,26*). These agents may function similarly to those identified in the above model systems or by other indirect pathways (Fig. 2). They may either directly or indirectly stimulate the secretion of growth factors, which in turn stimulate the growth of an initiated cell. An example of

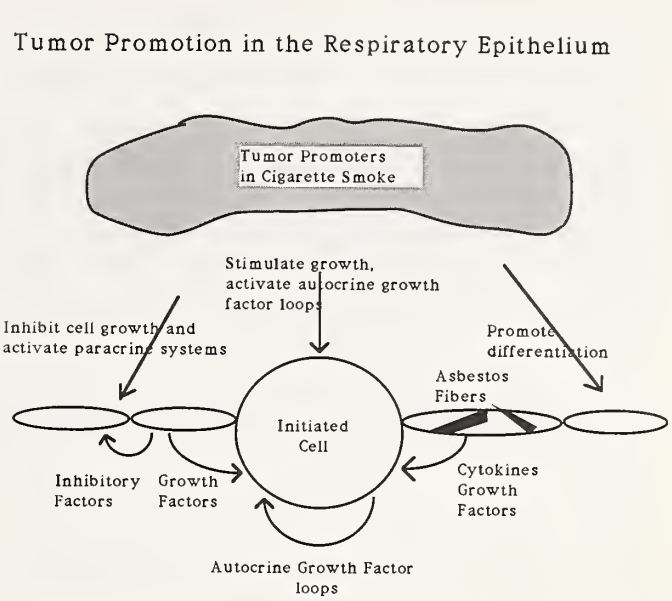

Fig. 2. Biologic mechanisms involved in tumor promotion in the lung.

this may be asbestos, which functions in the lung as a tumor promoter. It is well established that asbestos is epidemiologically associated with an increased risk for lung cancer. However, asbestos fibers appear to be chemically inert and have not been shown to interact with DNA directly or with cells via specific biochemical pathways. Instead, asbestos remains in the lung or pleural cavity for years, where the fibers are phagocytized by macrophages potentially stimulating the release of cytokines and growth factors. This, in turn, may mediate a promotion-like effect on the respiratory epithelium (Fig. 2).

MOLECULAR MECHANISMS OF TUMOR PROMOTION

The application of molecular biology to the process of tumor promotion has begun to identify potential mechanisms by which these agents function. These mechanisms must explain two major biologic characteristics of these agents: 1) a wide variety of factors and chemicals are able to enhance tumor formation even within a single tissue, and 2) all tumor promoters are characterized by their reversible biologic effects. Recent biochemical evidence suggests that tumor promoters function by binding to or affecting gene products (as opposed to mutating DNA directly), which in turn affect gene regulation. Thus, their biologic effects depend upon a continual interaction between the tumor promoter and cellular proteins. This would conveniently explain the reversible nature of these agents, the need for multiple applications, and the fact that promoter treatment preceding initiator application has little effect. In addition, there appears to be a plethora of cellular proteins that can serve as substrates (explaining the wide diversity of tumor promoters), all of which ulti-

mately feed into common pathways affecting cell growth and differentiation.

Model systems have shed significant light on how this process works. Tumor promoters such as phorbol esters have been shown to bind and activate protein kinase C, a major enzyme involved in signal transduction from the cell membrane (*27,28*). This enzyme transmits signals to the cell by affecting the expression of multiple downstream genes. It was observed several years ago that TPA-responsive genes have a similar nucleic acid sequence in their promoter regions (TGACTCA), named the TPA response element (TRE) (*29*). It was hypothesized that a transcriptional complex would bind to this element and stimulate transcription of these genes. Simultaneously, several groups independently identified this complex (called AP-1) and demonstrated that it contained multiple, separate transcription factors (*30–32*)). Prominent among these are the jun and fos gene family members. These genes are early-response genes whose expression is rapidly increased after stimulation with growth factors (*33*). They encode phosphoproteins (c-jun, c-fos), which are able to heterodimerize and specifically bind DNA and regulate gene expression. Indeed, c-jun and c-fos bind to a DNA sequence that is identical to TRE. Thus, these proteins supply the link between the signal transduction pathway triggered by TPA and the regulation of downstream genes mediating the biologic effects of TPA. In fact, it has recently been shown that TPA induces the dephosphorylation of c-jun, which increases its affinity for DNA and hence produces increased DNA binding and transactivation of genes (*34*).

The role of c-jun in mediating the promoting effects of TPA is perhaps best seen in an in vitro assay of the cotransforming activity of TPA. Previous results demonstrated that rat embryo cells, which were initiated by transfection with an activated c-Ha-ras gene, could be fully transformed by treatment with TPA (*35*). Interestingly, we have recently shown that deregulated expression of c-jun will substitute in this system for TPA in cotransforming rat embryo cells (*36*). In addition, recent work suggests that c-jun cotransformation activity requires specific regions of the protein including the DNA binding, dimerization, and transactivation regions (unpublished data). Thus, c-jun appears to cotransform cells by the transactivation of downstream genes.

Although most of the systems described above are TPA-induced, it is reasonable to assume that as early-response genes, c-jun and c-fos mediate signals from many different receptor-ligand combinations. Thus, they (and other transcription factors) may play a critical role in mediating the biologic effects of a wide range of factors that could function as tumor promoters. While the identity and nature of tumor promoters remain unknown in the respiratory epithelium, it is clear that potential candidates function in part by inducing cell proliferation either by direct action at the cell membrane or indirectly via the production of a known growth factor. The signal pathways that allow multiple factors to stimulate growth in these cells remain essentially unknown. Recent work has begun to identify the transcription factors mediating these signals in respiratory epithelial cells (Figs. 3 and 4). It is clear that in both primary cells and their transformed counterpart (H345 small-cell, lung-cancer cell line), c-jun and c-fos are expressed and are exquisitely sensitive to stimulation from growth factors. This type of response can be obtained from a wide range of individual growth factors, suggesting that these independent growth factor pathways converge into one involving, in part, c-jun and c-fos. In addition, the inducibility of these genes is directly related to the sensitivity of these cells to growth factors. Cell lines (myc amplified), which are rapidly growing, have a blunted or absent serum stimulation of c-jun and c-fos, while slower growing cells have a much stronger induction of the expression of these genes (data not shown). Whether these transcription factors mediate actual tumor promotion in respiratory epithelium remains to be determined, but their identification and inducibility with growth factor stimulation makes them prime candidates.

IMPLICATIONS FOR EARLY DIAGNOSIS AND PREVENTION

The elucidation of the multistage nature of epithelial cancers has now provided a rational approach for the early diagnosis and potential prevention of these cancers. If early genetic events (such as an activating mutation in a ras gene) can be identified, then these events can be used

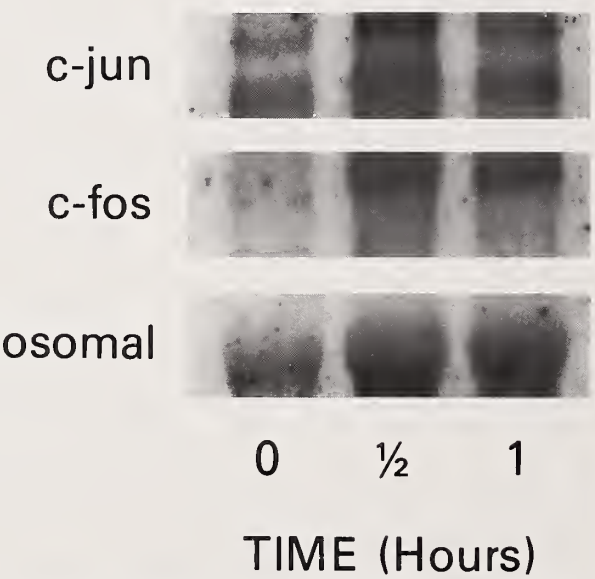

Fig. 3. c-jun and c-fos mRNA expression in primary bronchial epithelial cells after growth factor stimulation. Cells were isolated and established in short-term culture and then exposed to minimal media for 18 h, after which complete growth factor containing media was added and total RNA isolated as described previously (*34*) at the time points shown. Separated in agarose was 10 μg of total RNA and transferred to nitrocellulose and hybridized to 32-P labeled probes as described previously (*32*).

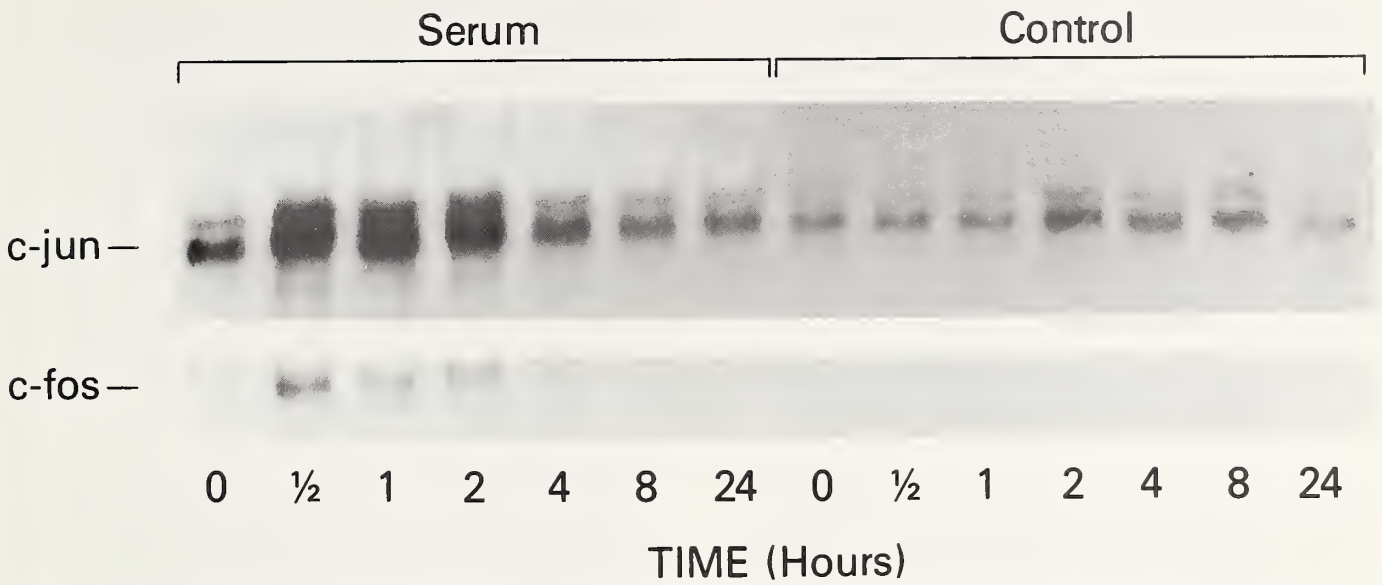

Fig. 4. c-jun and c-fos mRNA expression in small-cell lung cancer cell line H345 after serum stimulation. Cells growing in RPMI-1640, supplemented with 2% serum, were starved for 48 h and then stimulated with the addition of serum to a final concentration of 2%. Total RNA was isolated at the listed time points and processed as in Fig. 3. Control cells were treated in similar fashion without the addition of serum. All lanes contained similar amounts of RNA as determined by ethidium staining (data not shown).

to carefully and specifically identify initiated cells. In the lung, it is conceivable that sputum specimens from high-risk patients could be screened for the presence of gene mutations by the polymerase chain reaction coupled with direct nucleic acid sequencing. Not only is this technically feasible, it is likely to be more sensitive and reproducible than present screening approaches. However, this approach is critically dependent on a careful identification and delineation of the precise chronology of the early activation events during the development of lung cancer. Once established, it is possible that a battery of genetic lesions in a defined set of genes could be used as a screening panel to identify preneoplastic lesions.

Prevention must be focused on the two critical elements in the development of early cancers—initiation and promotion. The genetic nature of initiation makes it less amenable to manipulation because it is irreversible. One approach, of course, is to eliminate the carcinogens that induce DNA damage, but this remains more a social issue than a medical one. An alternative is to devise methods of inactivating the intact gene product, such as using antisense RNA or DNA. Finally, one could attempt to interfere with the downstream biochemical pathways initiated by activated oncogenes, which may well neutralize the effects of initiation. However, the inherent reversible nature of tumor promotion makes it a better target for intervention to prevent the development of malignancy. Since this may be mediated through specific growth-factor stimulation, one approach would be to interfere with growth-factor function by use of growth-factor-specific antibodies or antagonists. However, since multiple growth factors may be functioning simultaneously, it may be wiser to block their effects further downstream, at the level of the transcription factors they influence.

Recent work has provided some data on how this blockage might be accomplished. c-jun and c-fos combine in the transcriptional complex AP-1, which is a master regulator of gene expression and has been shown to mediate the biologic effects of at least one tumor promoter, TPA. It is now clear that there is cross-talk between transcriptional systems, which allows for modulation of transcriptional activation. Several proteins have recently been described that interact with the AP-1 complex to alter its activity (*37–39*). These include the glucocorticoid receptor, estrogen receptor, and retinoic acid receptor (*37,38*). In addition, there are other characterized and uncharacterized transcription factors and proteins that are able to complex with c-jun or c-fos and affect their ability to transactivate genes (*39*). These factors and the pathways that activate them may be important counterregulatory systems to AP-1, which in turn may abrogate the biologic effects produced by AP-1 transcriptional systems. For instance, in inflammed joints, synovial cells express high levels of AP-1 responsive genes such as collagenase, which mediate joint destruction (*37*). Treatment with glucocorticoids is a standard, effective way to inhibit joint inflammation and, in part, may relate to the blockage of AP-1 transactivation and down regulation of genes such as collagenase. The retinoic-acid receptor is of particular interest since retinoic acid has long been known to promote epithelial cell differentiation. In addition, a recent clinical trial has shown that it is an effective chemopreventive agent for head and neck squamous-cell carcinomas and possibly lung carcinomas (*40*). It is conceivable that retinoic acid effectiveness extends in part from its ability to cross talk with AP-1 or other transcriptional complexes.

The advantage of these agents (which affect transcriptional complexes) is that they would be interacting with

converging biochemical pathways, which in turn would potentially enable them to block multiple discrete signals from the cell surface. The identification of appropriate agents then becomes the major task. This can be accomplished in two fashions: 1) Identify and characterize the specific tumor promoters and their pathways in respiratory epithelium. One could then rationally design agents that would interfere with those pathways. 2) Design in vitro or in vivo assay systems for tumor promotion and screen for agents that interfere with that activity. Such systems have recently been described and have yielded interesting results (*41*). Once candidate agents are identified, they can then be tried in preclinical animal testing and ultimately in human clinical trials.

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myc Family DNA Amplification in Tumors and Tumor Cell Lines From Patients With Small-Cell Lung Cancer

Bruce E. Johnson, John F. Brennan, Daniel C. Ihde, Adi F. Gazdar[1,2,3]

ABSTRACT—The myc family DNA copy number of 291 specimens (183 tumors and 108 tumor cell lines) from patients with small-cell lung cancer has been reported in 15 different studies. Thirty-five of 108 (32%) cell lines from small-cell lung cancer patients have myc family DNA amplification (16 c-myc, 7 N-myc, and 12 L-myc). Thirty-seven of 183 (20%) tumors from patients with small-cell lung cancer have myc family DNA amplification (3 c-myc, 13 N-myc, and 18 L-myc). The myc family DNA copy number in tumors from patients with small-cell lung cancer is similar in the majority of sites from the same patient. The presence of myc family DNA amplification in the tumor cell line is also typically present in the tumor from the same patient. myc family DNA amplification is present in a minority of patients with small-cell lung cancer, and the data on its association with shorter survival of patients are meager at present. Future studies on the biology of the myc family in small-cell lung cancer may require use of newer technologies that can work with small tissue samples typically available at the start of therapy. [J Natl Cancer Inst Monogr 13:39–43, 1992]

Three of the members of the myc family, c-myc, N-myc, and L-myc, have been shown to be amplified in tumors and tumor cell lines from patients with small-cell lung cancer (*1–15*). c-myc amplification is associated with the variant form of small-cell lung cancer cell lines (*1,16,17*). The variant cell lines typically have a more rapid growth rate and a higher cloning efficiency in soft agarose, and they lack markers of neuroendocrine differentiation (L-dopa decarboxylase and bombesinlike immunoreactivity) when compared to the more common classic small-cell lung cancer cell lines (*17*). In previously published reports we have shown that myc family DNA amplification is more common in tumors and tumor cell lines derived from small-cell lung cancer patients previously treated with combination chemotherapy (*9,18*). Furthermore, DNA amplification of c-myc in tumor cell lines established from chemotherapy-treated patients is associated with a shortened survival time (*18*).

We wanted to compare our observations of the frequency of myc family DNA amplification in tumors and tumor cell lines from patients with small-cell lung cancer to other published series. In addition, we wanted to compare our observations about the clinical situation of patients with small-cell lung cancer whose tumors and tumor cell lines had myc family DNA amplification to other published series. Therefore, we reviewed reports that studied four or more tumors or tumor cell lines from patients with small-cell lung cancer and the myc family DNA copy number. We report here on the comparative results of these studies and the support they provide to the observations from our previously published series of small-cell lung cancer cell lines and tumors.

MATERIALS AND METHODS

We studied tumors and tumor cell lines from 90 of the 306 patients with small-cell lung cancer treated at the National Cancer Institute-Navy Medical Oncology Branch from 1977 through 1986. The patients underwent staging evaluations and treatment as we described in a previously published report (*15*). The myc family DNA copy number was determined (*9,15*). Specimens were identified as having been obtained prior to the initiation of chemotherapy or after one or more courses of combination chemotherapy.

Reports of myc family DNA amplification in at least four tumors and/or tumor cell lines from patients with small-cell lung cancer were identified by a MEDLINE search of the English literature. One cell line per patient is included in Table 1 if multiple cell lines were established from a single patient. The clinical situations of the patients from whom the tumor cell lines were established or from whom tumors were obtained were reviewed if available and compared to our previously published reports (*9, 15,18*).

RESULTS

NCI-Navy Medical Oncology Branch Results

A total of 107 small-cell lung tumors and tumor cell lines from 90 patients was evaluated for myc family DNA amplification (*15*). There were 38 tumor specimens and 69 tumor cell lines. These were obtained from 66 patients

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Table 1. myc family DNA amplification of small-cell lung cancer cell lines

Ref. no.	No. of cell lines with myc family DNA amplification		
	c-myc	N-myc	L-myc
2	2/4 (50%)	0/4 (0%)	Not done
6	0/6 (0%)	0/6 (0%)	Not done
7	4/5 (80%)	0/5 (0%)	Not done
8	0/9 (0%)	3/9 (33%)	3/9 (33%)
12	3/15 (20%)	0/15 (0%)	4/15 (27%)
15	7/69 (10%)	4/69 (6%)	5/69 (7%)
Total	16/108 (15%)	7/108 (6%)	12/93 (13%)

with extensive-stage disease and 24 patients with limited-stage disease. Sixty-seven specimens were obtained from patients initially treated with the combination chemotherapy. Forty specimens were obtained from patients who had not received chemotherapy. Nineteen (28%) of the 67 tumors and tumor cell lines from previously treated patients had DNA amplification of one of the myc family genes (seven c-myc, seven N-myc, and five L-myc). In contrast, only three of 40 specimens obtained from patients who had not received chemotherapy had myc family DNA amplification (one N-myc and two L-myc; $P = .01$ by the chi-square test).

myc Family DNA Amplification in Small-Cell Lung Cancer Cell Lines

We were able to identify 10 different publications reporting myc family DNA amplification in small-cell lung cancer cell lines between 1983 and 1991. The first report was by Little et al in 1983. We have not included the multiple results of the same tumor cell lines if they are reported in more than one study. Six different studies have studied a total of 108 cell lines with 4–69 tumor cell lines in each series (Table 1). c-myc and L-myc DNA amplification are present in 15% and 13%, respectively, of the tumor cell lines, and N-myc is present in 6%. No tumor cell line from a small-cell lung cancer patient has DNA amplification of more than one myc family gene except Lu-135, which has DNA amplification of both c-myc and L-myc (*11*).

Table 1 shows that four of the five series of small-cell lung cancer cell lines other than those reported by us (*15*) have myc family DNA amplification in a high percentage (47%–80%) of the tumor cell lines. The single exception was reported by Morstyn et al (*6*), where the cell lines did not have any myc family DNA amplification. Five of the six tumor cell lines in this series were established from patients with small-cell lung cancer who had not received any combination chemotherapy. This is consistent with our previous observation that myc family gene amplification is not as common in tumor cell lines established from untreated patients (*15*).

Three different reports (*7,12,19*) describe myc family DNA amplification in 14 tumor cell lines established from six patients with small-cell lung cancer at different times during their clinical course. In two of these studies (*7, 12*), the myc family DNA amplification status appears to be consistent in five of the patients. In the tumor cell lines established from the individual patients, the same myc family gene was amplified in each series of cell lines (c-myc in five cell lines from two patients, L-myc in four cell lines from two patients, and none in two cell lines from one patient). Kok et al (*19*) have reported an exception to this observation. They have described a series of three cell lines established from a single patient with small-cell lung cancer at presentation, at recurrence after nine cycles of chemotherapy, and after a subsequent course of radiotherapy. The tumor cell line established prior to the initiation of treatment had N-myc DNA amplification, and the two cell lines established after the patient's tumor recurred had c-myc DNA amplification. This is the single example where tumor cell lines established from the same patient at different times during the treatment course had a different myc family gene amplified.

myc Family DNA Amplification in Small-Cell Lung Cancer Tumors

We were able to identify seven different publications from 1986 to 1990 reporting myc family DNA amplification in tumors from 183 patients with small-cell lung cancer. The seven different studies have reported the myc family DNA amplification of tumors from 5 to 47 patients with small-cell lung cancer (Table 2). The proportion of myc family DNA amplification in each series of 10 or more patients is very similar. The percentage of patients with c-myc DNA amplification in their tumor is 3% (range 0%–9%), the percentage with N-myc is 8% (range 0%–11%), and the percentage with L-myc is 13% (range 5%–18%).

Two studies (*5,12*) showed that the myc family DNA copy number was similar in multiple sites of metastatic disease from 26 different patients with small-cell lung cancer (Table 3). Wong et al (*5*) reported on four patients in whom all 10 metastatic lesions had amplified copies of the myc family genes (two patients with c-myc and two with N-myc DNA amplification). Additionally, in 17 patients the original tumor did not have myc family DNA amplification, nor did their 41 metastatic lesions. Takahashi et al reported on 11 simultaneous tumor sam-

Table 2. myc family DNA amplification in tumors from patients with small-cell lung cancer

Ref. no.	No. of tumors with myc family DNA amplification		
	c-myc	N-myc	L-myc
5	2/45 (4%)	3/45 (7%)	Not done
9	0/38 (0%)	4/38 (10%)	2/38 (5%)
10	0/5 (5%)	0/5 (0%)	3/5 (60%)
11	0/17 (0%)	1/17 (6%)	3/17 (18%)
12	2/23 (9%)	0/23 (0%)	3/23 (13%)
13	1/8 (13%)	0/8 (0%)	2/6 (33%)
14	1/47 (2%)	5/47 (11%)	5/47 (11%)
Total	6/183 (3%)	13/183 (7%)	18/136 (13%)

Table 3. myc family DNA amplification pattern in multiple tumors from the same patient with small-cell lung cancer

Ref. no.	Same myc family DNA copy numbers	Different myc family DNA copy numbers
5	21/21	0/21
11	0/1	1/1
12	5/5	0/5
14	9/11	2/11
Total	35/38 (92%)	3/38 (8%)

ples in five patients with small-cell lung cancer (*12*). One patient's tumor samples had c-myc, one patient's samples had L-myc, and three patients' samples had no myc family DNA amplification. In addition, one patient with small-cell lung cancer had c-myc DNA amplification in the tumor before treatment and at postmortem examination.

In contrast, two other reports have described different myc family DNA copy numbers in different sites from the same patient with small-cell lung cancer. Yokota et al studied the primary tumor and four different sites of metastases in a patient with small-cell lung cancer (*11*). The primary tumor, the pulmonary hilar lymph node, and the pleural metastasis all had about 100-fold amplification of N-myc, whereas a liver metastasis and a para-aortic lymph node did not have N-myc DNA amplification. Noguchi et al were able to study tumors from 47 patients with small-cell lung cancer (*14*). Eleven patients had myc family DNA amplification in their primary tumor. Nine of the 11 had similar degrees of myc family DNA amplification in the metastatic sites as in the primary tumor site. One of the other two patients had N-myc DNA amplification in part of the primary tumor and three metastatic sites, whereas a different part of the primary tumor and three metastatic sites did not have N-myc DNA amplification. The other patient had L-myc DNA amplification in metastatic lymph nodes present at postmortem examination, but amplification was not present in the primary lesion that had been resected prior to the patient's death. No investigators found DNA amplification of multiple members of the myc family DNA in the same sample.

myc Family DNA Amplification in Tumors and Tumor Cell Lines From the Same Patient with Small-Cell Lung Cancer

We had previously published our study showing that the myc family DNA copy number is similar in tumors and tumor cell lines from 16 of 17 evaluable patients with small-cell lung cancer (*15*). The single exception was in a patient in whom the tumor DNA had degraded, so the DNA copy number of the tumor could not be accurately determined. Information is also available from other investigators. Yokota et al reported that a cell line, Lu-135, was amplified for both c-myc and L-myc (*11*). The tumor from which Lu-135 was established had L-myc DNA amplification but did not have c-myc DNA amplification. They also reported on a second tumor cell line, Lu-139, which had c-myc DNA amplification, whereas the original tumor did not. Takahashi et al reported on two patients with small-cell lung cancer who had both their tumors and their tumor cell lines studied (*12*). One patient had c-myc DNA amplification in two tumor samples as well as two tumor cell lines established from these tumor specimens. The other patient did not have myc family DNA amplification in either the two tumor cell lines or the tumors. Kok et al reported on the myc family DNA amplification status of a tumor and a tumor cell line established from the same patient prior to starting his therapy (*19*). The supraclavicular lymph node and the tumor cell line established from it, GLC-14, had N-myc DNA amplification. In contrast, the tumor cell lines established from tumors after treatment, GLC-16 and GLC-19, had c-myc DNA amplification, and the tumors were not available for study.

DISCUSSION

Most investigators who have studied 4–15 tumor cell lines established from different patients with small-cell lung cancer have found myc family DNA amplification in 47%–80% of the cell lines (*2,7,8,12*). In contrast, we have found myc family DNA amplification in only 16% of the 69 tumor cell lines we have studied (*15*). We believe the low percentage of myc family DNA amplification observed in our tumor cell lines occurs in part because an increased proportion of the tumor cell lines analyzed are established from previously untreated patients. In the other series where the treatment status of patients with small-cell lung cancer from whom the tumor cell lines were established was reported, five of the six patients had not received combination chemotherapy. None of these six tumor cell lines had myc family DNA amplification, supporting our observation that myc family DNA amplification is less common in tumor cell lines established from untreated patients with small-cell lung cancer.

In contrast to the relatively high incidence of myc family DNA amplification in most series of tumor cell lines from patients with small-cell lung cancer, studies of 17–47 tumors obtained from different patients have found myc family DNA amplification in only 11%–24% (*5,9,11,12, 14*). The most marked difference in the percentage of myc family DNA amplification between the tumor and the tumor cell lines is in c-myc. Three percent of the tumors had c-myc DNA amplification compared to 15% of the tumor cell lines.

Four examples of c-myc DNA amplification have been described in a tumor cell line that was not present in the original tumor from the patient (*11,15,19*). In one patient the DNA was degraded in the small-cell lung cancer specimen, causing the c-myc DNA copy number to be underestimated, while the tumor cell line from the same patient had c-myc DNA amplification (*15*). Other potential explanations for observing myc family DNA amplification in tumor cell lines while the original tumor does not have myc family DNA amplification include the following: 1) the tumor may have a mixture of normal and tumor cells in it, making it more difficult to detect low levels of amplification, or 2) a subpopulation of cells that have

myc family DNA amplification may grow out in cell culture and become the predominant cells present. These explanations may be responsible for the increased frequency of c-myc amplification seen in tumor cell lines and compared to tumors from patients with small-cell lung cancer. This hypothesis is also supported by the observation that c-myc amplified cells have a higher cloning efficiency and faster growth rate and that transfection of the c-myc gene into a tumor cell line that did not previously express c-myc mRNA is associated with a faster growth rate and higher cloning efficiency (*17*). In contrast to c-myc, the percentage of tumors and tumor cell lines that have N-myc DNA amplification (6% vs. 7%, respectively) and those having L-myc DNA amplification (13% vs. 13%, respectively) is remarkably similar (Tables 1 and 2).

The DNA amplification of myc family genes has been associated with shortened survival of patients with small-cell lung cancer in two different studies (*14,15*). Our study of cell lines established from previously treated patients with small-cell lung cancer showed that the c-myc DNA amplification is associated with shortened survival (*15*). In addition, Noguchi et al (*14*) reported an association between patients with advanced-stage small-cell lung carcinoma with 10 or more copies of one of the myc family genes in their tumors and shortened survival. However, both of the studies used samples taken from patients after the initiation of therapy, so their applicability to samples taken prior to therapy remains unexplored.

The incidence of myc family DNA amplification in tumors studied thus far from patients with small-cell lung cancer is low even among patients previously treated with combination chemotherapy. In addition, our experience in collecting specimens from untreated patients with small-cell lung cancer suggests that it will be difficult to obtain adequate fresh tissue specimens for routine Southern blot analysis, which looks for myc family DNA amplification. Therefore, the routine determination of myc family DNA amplification in tumor specimens from patients with small-cell lung cancer analogous to N-myc DNA amplification determination in tumor specimens from patients with childhood neuroblastoma does not appear to be warranted (*20*).

The avenues of research about the myc family in small-cell lung cancer and their potential impact on survival that still remain to be explored include myc family mRNA expression and protein production in tumor samples. Potentially useful studies should require less tissue than that needed for Southern blots. We suggest that future research on the role of the myc family in small-cell lung cancer include additional studies that do not rely solely on the DNA amplification as a measure of activation but include other tests that could include in situ hybridization and/or polymerase chain reaction, which looks for mRNA expression, and immunohistochemistry, which looks for protein production.

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Animal Models for Chemoprevention of Respiratory Cancer[1]

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ABSTRACT—Of the several models for lung carcinogenesis, two appear appropriate for chemoprevention studies based upon dose response, tumor type, and tumor localization. One model utilizes the direct-acting carcinogen methylnitrosourea (MNU), and the other utilizes a carcinogen (diethylnitrosamine) requiring metabolic activation. Tumors appear rapidly in both models (within 6 months), and the model systems are responsive to modulation by several classes of potential chemopreventive agents. For example, the retinoid *N*-(4-hydroxyphenyl) retinamide reduces the incidence of lung adenosquamous carcinoma, but retinol or beta-carotene are ineffective when administered alone. However, concomitant administration of these compounds reduces the incidence of non-neoplastic dysplasias as well as adenosquamous carcinomas of the lung. In the MNU system, retinoids in general have been ineffective in reducing the incidence of tracheobronchial squamous-cell carcinomas. [J Natl Cancer Inst Monogr 13:45–49, 1992]

Two animal models for lung cancer that permit the rapid evaluation of potential chemopreventive agents have been used in our laboratory. The requirements for these models were that they reproducibly induce a high incidence of respiratory cancers within a short period of time (6 months), produce neoplastic lesions in relatively localized areas so that histological processing would be kept to a minimum, be simple enough that large numbers of animals could be treated, and have little, if any, toxic effect.

Previous attempts to establish a lung cancer model that is histologically and biochemically similar to bronchogenic carcinoma in man have had limited success (*1,2*). Earlier studies employed methods such as thread transfixions (*3,4*) and exposure to radioactive compounds, either by inhalation (*5*) or implantation of intrabronchial pellets (*6*). Lung cancer models employed by Harris et al (*7,8*) and Saffiotti and associates (*9,10*) required the intratracheal instillation of suspensions of a crystalline polycyclic aromatic hydrocarbon into carrier particles of inert dust. Nitroso-compounds, with and without carrier dust, to induce tracheal and pulmonary lesions have also been employed (*11–15*). However, the physiochemical properties of these carcinogens, such as solubility and particle size, and the nature of the carrier particles are critical to tumor induction. As a result, the feasibility of obtaining a reproducible incidence of respiratory cancer in different laboratories by use of these techniques appears remote. Furthermore, many of the earlier lung tumor models exhibited a very low cancer incidence and a long latency period.

LUNG CANCER MODEL (*N*-NITROSODIETHYLAMINE/SYRIAN GOLDEN HAMSTER)

The organ-specific carcinogenicity of *N*-nitrosodiethylamine (DEN) for the respiratory tract of Syrian golden hamsters was demonstrated by several studies (*16,17*), including those from our laboratory (*18*). Regardless of the route of administration of DEN, tumors developed predominantly in the trachea, oral cavity, and larynx, accompanied by a much lower incidence of tumors of stem bronchi and peripheral lung. The high sensitivity of trachea to DEN carcinogenesis was shown by Dontenwill (*19*) using an intrasplenic implantation technique. More recent biochemical studies showed that tracheal tissue metabolized DEN efficiently with the formation of ethylated DNA adducts (*20,21*).

A dose-response correlation for the induction of upper respiratory tract tumors was demonstrated by Montesano and Saffiotti (*17*) in hamsters given subcutaneous injections at dose levels of 0.5, 1, 2, and 4 mg of DEN once weekly for 12 weeks (total dose: 6 to 48 mg per hamster). Depending on the dose, tumors developed as early as 11 weeks, and the incidences ranged from 88% to 100% in the trachea to 17% to 75% in the nasal cavities and the larynx. Only three tumors of the lung were observed in a total of 142 animals. The majority of the tracheal tumors were papillomas. The malignant potential of these tumors was demonstrated by transplantation experiments.

The pathogenesis of DEN-induced neoplasms of the respiratory tract was investigated (*22,23*) by serial sacrifice of Syrian golden hamsters injected subcutaneously with a high dose of DEN (17.8 mg/kg; 0.1 LD_{50}), twice weekly for 20 weeks (total dose: 72 mg/100 g). This dose schedule, in contrast to the above regimens, yielded a tumor incidence of 90% to 100% in the trachea and 60% to 70% in the lung when the study was terminated at 24 weeks (*24*). Similar tumor incidence was observed in our studies using the same experimental protocol. Serial sacrifice

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studies by Reznik-Schuller (*23*) showed that the lung tumors originated primarily from the pulmonary clara cells and endocrine cells, whereas the tracheal tumors were derived from the basal cells. Since lung tumors with features of pulmonary endocrine cells (small-cell carcinoma) account for 20% to 30% of all lung cancer, the DEN hamster model appeared to be a suitable system for evaluating potential chemopreventive agents against respiratory tract cancer.

TRACHEOBRONCHIAL CANCER MODEL (*N*-METHYL-*N*-NITROSOUREA/SYRIAN GOLDEN HAMSTER)

The induction of invasive cancers in a localized area of the hamster trachea by the carcinogen *N*-methyl-*N*-nitrosourea (MNU) was originally described by Schreiber et al. (*25*), but we have substantially modified both the equipment and the methodology for use in chemoprevention studies (*26*). This model for respiratory cancer eliminates many of the problems inherent in the benzo(a)pyrene/ferric oxide model for lung tumor induction in hamsters. Through the use of a specially designed catheter, the area of tissue exposed to MNU is limited to a defined region of the trachea; tumors develop only in the exposed area of the trachea. Because organ exposure is limited to a known area, quantitation of doses is facilitated. MNU is delivered and reabsorbed by the catheter using no carrier particles; thus, any possible influence of particle size, composition, or method of carcinogen/carrier preparation is eliminated. Studies completed in this laboratory have developed dosing regimens that yield a reproducible tumor response, with little or no toxicity. Serial kill studies have characterized the histogenesis of the carcinogen-induced lesions (*27*), as well as provided a relevant time frame for the development of MNU-induced tracheal cancer. The lesions show a characteristic histogenic pattern of goblet-cell hyperplasia and loss of ciliated cells, followed by squamous metaplasia with increasing stratification, carcinoma in situ, and invasive carcinoma (*28*).

The staff at IIT Research Institute redesigned and fabricated a catheter that, although similar to that described by Schreiber et al. (*25,29*), has been modified for use in studies of chemoprevention. The catheter is designed to deliver a predetermined quantity of carcinogenic solution followed by reabsorption of the carcinogen; the area of carcinogen exposure is limited to a 6-mm length of the trachea 10 mm to 16 mm distal to the vocal cords. The use of vital dyes and development of tumors have confirmed that carcinogen exposure is limited to this area of the trachea.

The influence of various MNU dosing regimens on animal toxicity and incidence of tracheal cancer has been detailed in two publications (*26,27*) from our laboratory and demonstrated in Fig. 1. As a result of these studies, treatment of animals once weekly with a solution of 0.5% MNU appears to be optimal for studies of inhibition of

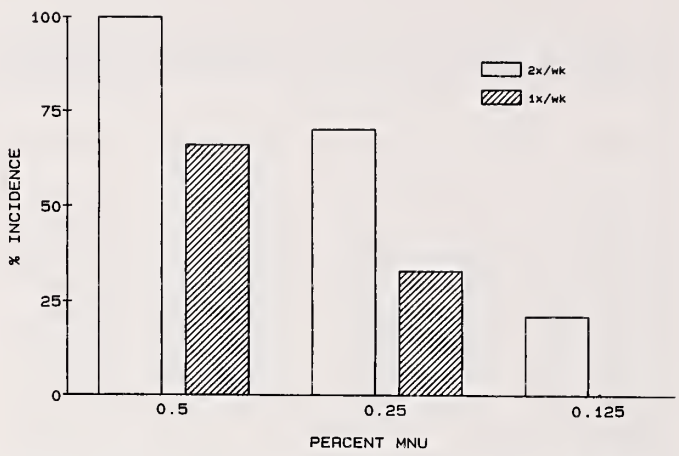

Fig 1. Effect of concentration of MNU and number of instillation on incidence of tracheal squamous cell carcinomas induced in the Syrian golden hamster. MNU was administered once or twice weekly for 15 wks by intratracheal instillation. All hatched bars significantly different from corresponding concentration open bars ($P < .05$).

carcinogenesis. Weekly administration at higher concentrations of MNU (1.0%) was found to be toxic, as was instillation of 0.5% MNU twice per week. Desired cancer incidence levels can be obtained by varying the length of MNU treatment; administration of 0.5% MNU once weekly for 15 weeks yields a cancer incidence of approximately 60% at 6 months, while shorter periods of MNU treatment yield fewer cancers. The dose-response curve for MNU-induced tracheal cancer appears to be approximately linear, from 0% to 75% cancer incidence.

RETINOID CHEMOPREVENTION OF RESPIRATORY CANCER

The rationale for the use of retinoids as chemopreventive agents or inhibitors of carcinogenesis originated with the studies of Mori (*30*), when it was observed that a deficiency of vitamin A led to metaplastic changes of the epithelium of the respiratory tract. The normal ciliated columnar epithelium of the trachea became flattened, lost nuclei, and became cornified, and the underlying layer of cells exhibited typical keratohyaline granules. The remarkable observations of Mori on the development of such retinoid-deficient squamous metaplasia indicated a process closely akin to that induced by certain chemical carcinogens (*27,31*). A more direct link between retinoids and cancer appeared in 1926 when Fujimake (*32*) observed the development of carcinomas of the stomach in rats maintained on a vitamin A-deficient diet. Other investigators have also shown that animals fed a diet deficient in retinoids and subsequently exposed to chemical carcinogens developed a greater-than-normal incidence of respiratory cancers as well as putative precursors to these malignancies (*33,34*).

Table 1. Effect of 4-HPR on the incidence of lung adenosquamous carcinomas induced in Syrian golden hamsters with DEN

No. of animals	DEN	Chemopreventive agent*	No. of animals with cancer	Percent incidence
29	+	None	10	34.5
30	+	4-HPR	4	13.3†
29	+	Se	5	17.2
29	+	Se + E	5	17.2
29	+	4-HPR + Se + E	1	3.5‡

* Dietary administration of Se = sodium selenite; E = DL-α-tocopherol.
† Significantly different from group 1, $P<.05$.
‡ Significantly different from group 1, $P<.01$.

In addition to the relationship between retinoid deficiency and neoplasia, other studies have indicated that retinoids can reverse premalignant changes (*35,36*), suppress malignant transformation (*37*), and inhibit tumor promotion (*38*). Inasmuch as retinoids inhibit these aspects of the carcinogenic process, several investigators have extended these studies to show that exogenous retinoids can inhibit tumor formation in vivo at several different organ sites.

Saffiotti and co-workers (*39*) were the first to describe an inhibitory effect of retinoids on respiratory carcinogenesis. However, studies of the inhibition of chemically induced tumorigenesis of the respiratory tract by retinoids have been equivocal and, in some cases, contradictory. In the study by Saffiotti et al., tumors were induced in hamsters by the intratracheal instillation of benzo(a)pyrene adsorbed onto ferric oxide particles (3 mg benzo(a)pyrene plus 3 mg ferric oxide suspended in 0.2 mL saline) once a week for 10 weeks. Treatment with retinyl palmitate markedly reduced both the incidence and number of respiratory tumors. However, retinyl acetate was ineffective in inhibiting development of lung tumors induced by the Saffiotti technique. Nettesheim et al (*40*), on the other hand, showed that retinyl acetate inhibited metastic lung nodules induced in rats by 3-methylcholanthrene. A study by Port et al (*41*) showed bronchial carcinoma induced by the Saffiotti procedure to be effectively inhibited by 13-*cis*-retinoic acid, although enhancement of MNU-induced tracheobronchial carcinogenesis has been reported with 13-*cis*-retinoic acid, ethyl retinamide, and *N*-(4-hydroxyethyl)retinamide by Stinson et al (*42*). In an early study, *N*-(4-hydroxyphenyl) retinamide (4-HPR) neither enhanced nor inhibited tracheobronchial carcinogenesis induced with MNU (*43*). More recently, however, we have found that 4-HPR is an effective inhibitor of DEN-induced lung carcinogenesis in the hamster (Table 1), but it has little effect on MNU-induced tracheobronchial carcinogenesis in this species. As indicated in the table, the combination of 4-HPR, selenium, and vitamin E was much more effective than when the agents were administered alone.

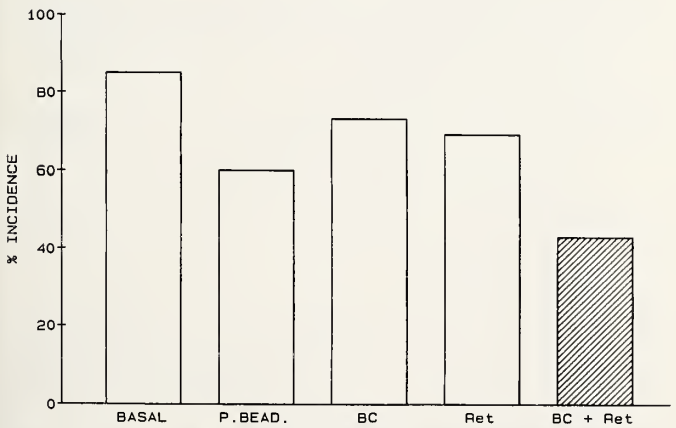

Fig 2. Effect of beta carotene (BC) alone or in combination with retinol (Ret) on incidence of lung adenosquamous carcinomas induced in male hamsters with diethylnitrosoamine. Beta carotene beadlets and retinol were administered as supplements to the basal diet (AIN-76A). Hatched bar significantly different from basal. P. Bead. = Placebo beadlets.

In the MNU model, neither selenium and vitamin E nor 4-HPR affect tracheal carcinogenesis; however, a combination of these three agents was very effective in inhibiting formation of squamous cell carcinomas of the trachea. Additional studies showed that both the natural retinoid, retinol, and the carotenoid, beta carotene, which is converted to retinol, are ineffective in suppressing respiratory carcinogenesis induced with either MNU or DEN. However, a combination of retinol and beta carotene inhibited lung adenocarcinoma development induced with DEN (Fig. 2). The reason for the synergistic effect of these two agents is presently unknown.

It is apparent from the experiments cited above that some natural and synthetic retinoids are highly effective in inhibiting respiratory carcinogenesis induced by several carcinogens. Moreover, in some cases the synthetic retinoids are more efficacious than the natural retinoids and are considerably less toxic. In those experimental systems (mammary and skin) that exhibit definite initiation and promotion stages of carcinogenesis, the retinoids have proven to be highly effective chemopreventive agents. If a definitive promotional phase is absent in an experimental tumor system, or is ill defined (tracheobronchial and lung), ambiguous results as to the chemopreventive effectiveness of the retinoids have been obtained, and in isolated cases the compounds may even promote carcinogenesis. Nevertheless, the overwhelming evidence indicates that retinoids inhibit carcinogenesis in such experimental tumor systems. From a clinical standpoint, the

additive or synergistic effect of combinations of agents may be important in reducing toxicity while increasing efficacy.

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Chemopreventive Studies in Barrett's Esophagus: A Model Premalignant Lesion for Esophageal Adenocarcinoma[1]

Harinder S. Garewal, Richard E. Sampliner, M. Brian Fennerty[2,3,4]

ABSTRACT—Barrett's esophagus is a premalignant lesion in which the lower esophagus is lined with metaplastic columnar epithelium rather than the normal stratified squamous epithelium. It is a precursor lesion for adenocarcinoma of the esophagus. We are studying Barrett's esophagus as a model premalignant lesion for adenocarcinoma from the standpoint of identifying biologic markers of increased cancer risk as well as therapeutic strategies for eradicating the lesion. Ornithine decarboxylase (ODC) activity in Barrett's mucosa was significantly higher than in normal adjacent mucosa from the same patient. However, polyamine content was not significantly altered, suggesting dysregulation of the polyamine pathway. Flow cytometry is being used to assess the presence of aneuploidy and its significance in a premalignant lesion. Initial results have demonstrated that aneuploidy and dysplasia can be discordant. Cytogenetic analysis using short-term epithelial cultures established from endoscopic biopsies of the lesion has demonstrated the presence of clonal karyotypic abnormalities. The clinical significance of aneuploidy and abnormal karyotype, however, remains to be proved. Chemopreventive intervention trials have included use of 13-*cis*-retinoic acid. Considerable toxicity was encountered, and the lesion showed no change in extent in 11 evaluable patients. A subsequent clinical trial with a biologic endpoint used α-difluoromethylornithine (DFMO), an irreversible inhibitor of ODC, to test whether a low dose could produce changes in polyamine content in gastrointestinal mucosa. Significant changes were observed in polyamine content after 6 weeks of DFMO treatment at 0.5 g/m^2 three times a day, suggesting that this clinically tolerable dose can result in measurable effects on the polyamine pathway. Additional strategies to be tested for lesion response include use of acid reflux suppression combined with "differentiating" agents. [J Natl Cancer Inst Monogr 13:51–54, 1992]

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Barrett's esophagus is a premalignant lesion for esophageal adenocarcinoma in which the esophagus is lined with metaplastic columnar epithelium rather than the normal stratified squamous epithelium (*1,2*). In the vast majority of cases, Barrett's esophagus is considered to be an acquired lesion resulting from chronic gastroesophageal reflux. However, neither the tissue nor the cell of origin of this lesion has been definitively identified. The clinical symptoms associated with Barrett's esophagus are those of gastroesophageal reflux and its complications, that is, heartburn, regurgitation, and dysphagia. Therefore, a definitive diagnosis can be made only by biopsy demonstration of metaplastic columnar epithelium in the esophagus.

Heartburn, the predominant symptom of reflux, is a frequent clinical complaint. Recent studies have shown that as many as 12% of patients with chronic heartburn who are evaluated by endoscopy and biopsy will have Barrett's esophagus (*3*). Hence, Barrett's is not an infrequent lesion. Furthermore, on the basis of autopsy prevalence compared with clinical recognition, Cameron et al have suggested that perhaps only one in 20 cases of Barrett's esophagus is recognized prior to death (*4*).

A high prevalence of Barrett's esophagus in patients who have received chemotherapy has been the subject of recent reports (*5*). In a prospective study of 16 women with breast cancer who received cyclophosphamide, methotrexate, and 5-fluorouracil chemotherapy, nine were found to have developed Barrett's esophagus (*5*).

The most serious complication of Barrett's esophagus is the associated increased risk of adenocarcinoma. Incidence studies have suggested a 30- to 40-fold increase in cancer risk, and prevalence data range from 8% to 40% occurrence of cancer (*2,6,7*). Hence, regular endoscopic and histologic surveillance is recommended for early detection of cancer.

Effective therapeutic options do not exist for patients with Barrett's esophagus. Aggressive antireflux measures, either medical or surgical, have failed to reproducibly eradicate the lesion. Because of its premalignant nature, prophylactic esophagectomy has been advocated for patients with high-grade dysplasia (*8*).

BARRETT'S ESOPHAGUS AS A MODEL PREMALIGNANT LESION

We are studying Barrett's esophagus as a model premalignant lesion for adenocarcinoma because it has

several unique characteristics. In contrast to the usual esophageal cancers that are of squamous histology, tumors arising in Barrett's esophagus are adenocarcinomas. Because the normal esophageal histology is squamous whereas the Barrett's epithelium is columnar, the latter is highly likely to be the tissue of origin of the adenocarcinoma. Adenocarcinomas account for about 10% to 20% of esophageal cancer, but the incidence appears to be increasing, with numbers of up to 40% being reported in recent series (*2*). Another feature of Barrett's esophagus is that it can be repeatedly and safely accessed by modern endoscopic techniques. Hence, the natural history of the lesion within an individual patient can be followed. This is in contrast to other premalignant lesions such as colonic polyps, which, once identified, are removed.

Because of these advantages we initiated our studies of this lesion, focusing on areas of potential clinical utility in chemoprevention. These include biologic characteristics that might be useful as intermediate markers of cancer risk as well as intervention trials with potential chemopreventive agents. Results of our studies are summarized below.

THE POLYAMINE PATHWAY

Ornithine decarboxylase (ODC) is the first enzyme in polyamine biosynthesis. It is an important enzyme in carcinogenesis, and its activity has been shown to be altered in some premalignant conditions in humans such as familial polyposis (*9–11*). Increased activity has also been reported in adenomatous colonic polyps (*12–14*). We have shown that ODC activity in endoscopic biopsies obtained from Barrett's mucosa was greater than that in normal uninvolved upper gastrointestinal (GI) mucosa obtained from the same patient (*15–17*). Preliminary observations based on a small cohort of patients suggest that ODC activity was greater in the presence of dysplasia in Barrett's esophagus than in its absence (*16*). We have further quantitated polyamine content in Barrett's mucosal biopsies to correlate this with the increased ODC. It is interesting that no relationship between either individual polyamine levels or total polyamines and the ODC activity could be demonstrated (*17*). This suggests that the regulation of the polyamine pathway may be different in this premalignant tissue than in normal cells.

The increase in ODC activity does not appear to be secondary to increased cellular proliferation. We have not directly compared labeling indices with ODC activity. Nevertheless, earlier studies suggest that the labeling index of Barrett's mucosa is not markedly increased over that of normal columnar upper GI mucosa, for example, gastric mucosa (*18*). In addition, in our studies we have found no relationship between ODC activity and percentage of cells in the S-phase of the cell cycle determined by flow cytometry.

FLOW CYTOMETRIC ABNORMALITIES

We and others have attempted to correlate flow cytometric analysis of DNA content with the presence or absence of dysplasia and carcinoma in Barrett's esophagus (*19–21*). Our results suggest that dysplasia can occur in the absence of aneuploidy and, conversely, the latter can be present without dysplasia. Reports by other investigators are in agreement with these results (*22*). This observation raises the possibility that aneuploidy may be a determinant of cancer risk independent of dysplasia or may precede histologic changes. Our long-term goal is to test whether risk in Barrett's esophagus is limited to patients with dysplasia and/or aneuploidy. The majority of patients, approximately 60%–75%, have neither aneuploidy nor dysplasia. If indeed the cancer risk is low in this group, then it may be possible to decrease or even discontinue surveillance endoscopies, thereby resulting in significant savings in medical care costs. Similarly, if a high-risk group could be identified, more intensive surveillance could be confined to this group, with the goal of early detection of cancer. Continued long-term follow-up of patients enrolled in ongoing studies is important to answer the question of clinical utility of detecting flow cytometric abnormalities.

TISSUE CULTURE OF EPITHELIAL CELLS FROM BARRETT'S MUCOSA

We have cultured epithelial cells from endoscopic biopsies of Barrett's mucosa (*23*). The cultured cells are incapable of colony formation in soft agar and are not capable of indefinite growth. Growth generally slows down at approximately passage 15. Hence, they do not behave as fully transformed cells.

The cultured cells have been used for cytogenetic analysis, performed at early passage. Clonal cytogenetic abnormalities have been identified, including somatic and sex chromosome numerical and structural changes (*24*). Multiple copies of chromosome 7 were identified in one patient in whom increased expression of epidermal growth factor receptor (EGFR) was demonstrated and shown to be the result of overexpression of EGFR mRNA (*25*). Clinical follow-up of these patients is continuing to determine whether the presence of karyotypic abnormalities will correlate with development of dysplasia or cancer.

The effect of potential chemopreventive agents on the growth of the cultured cells was studied using three different techniques: quantitation of cell number, colony formation on plastic surface, and an assay based on adenosine triphosphate (ATP) quantitation developed in our laboratory (*26*). In this in vitro system, retinoids and carotenoids (13-*cis*-retinoic acid, 4-(hydroxyphenyl)retinamide, beta-carotene, and canthaxanthin) did not significantly inhibit growth. In contrast, α-difluoromethylornithine (DFMO) resulted in significant growth inhibition, even at the lowest concentration tested (0.05 mM).

CLINICAL TRIALS

Clinical intervention trials with potential chemopreventive agents were begun at the same time that the

laboratory studies were initiated. No treatment for Barrett's esophagus has proved to be effective. The lesion does not reverse, even with aggressive medical or surgical antireflux therapy. Our first trial used 13-*cis*-retinoic acid, at a dose of 1 mg/kg per day, selected because of its activity in other premalignant lesions such as oral leukoplakia. As expected, considerable toxicity was noted at this dose, and only 11 of 16 patients were able to complete at least 6 weeks of treatment. There were no responses in extent or histology of the lesion in these 11 patients (*27*). In addition to the usual toxicities associated with this drug, two patients developed esophageal ulcers in their Barrett's mucosa, which healed on discontinuation of treatment (*28*).

The next trial used DFMO at a low dose of 0.5 g/m^2 three times a day. Although DFMO has considerable toxicity when used at its maximum tolerated dose of 4–6 g/m^2 per day—that is, the dose employed in trials for advanced malignancy—our objective was to test whether a low dose of DFMO, potentially usable in chemoprevention studies, would result in measurable changes in polyamine content in upper GI mucosa. Eight subjects were treated for 6 weeks with DFMO. Polyamine content was measured at baseline and at completion of treatment. Statistically significant changes in polyamine content were observed (approximately 60% decrease in spermidine-to-spermine ratio, resulting primarily from a decrease in spermidine content). These changes occurred in the Barrett's mucosa as well as in normal tissue from squamous esophagus, gastric, and small-bowel mucosa. This is the first demonstration that a low dose of DFMO can produce significant changes in the polyamine pathway in human GI mucosa. Additional trials of potential chemopreventive agents, either alone or in combination with antireflux agents, are planned.

In summary, Barrett's esophagus is a premalignant lesion with several unique advantages as a model for conducting laboratory and clinical studies. Knowledge gained from studying this lesion will increase our understanding of the carcinogenesis process and, it is hoped, will be of value not only in treating this condition but in cancer chemoprevention in general.

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An Animal Model for Oral Cancer

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ABSTRACT—Human head and neck squamous cell carcinogenesis (SCC) is a common malignancy that appears to be related to continuous exposure to putative carcinogens or promoters such as tobacco and alcohol. To understand the mechanisms of the development of head and neck cancer and to test the efficiency of new therapeutic approaches, the characterization of an animal model system is necessary. The cheek-pouch carcinogenesis model in Syrian golden hamsters is probably the best known animal system that is most closely comparable with the development of premalignant and malignant lesions in human oral cancer. Furthermore, it is one of the most well-characterized animal system models for SCC. Our first approach to understanding the cellular and molecular changes that occur in the hamster cheek-pouch carcinogenesis process was to compare this model to the mouse-skin system, in which a number of critical events have been well characterized. We examined the sequential expression of hyperplasia, micronucleated cells, ornithine decarboxylase activity, polyamine levels, transglutaminase I activity, epidermal growth factor receptor levels, expression of several keratins, γ-glutamyl transpeptidase, and nucleolar organizer regions. We suggest that these markers can be used to understand mechanisms of carcinogenesis and, in addition, can serve as alternative shorter end points in studies of chemoprevention. We also present preliminary molecular studies in the experimental oral model. We obtained a partial sequence of exon 2 of the Ha-ras gene and detected an $A^{182} \rightarrow T$ transversion in codon 61 in hamster cheek-pouch SCC induced by 7,12-dimethylbenz(a)anthracene. [J Natl Cancer Inst Monogr 13:55-60, 1992]

The cheek-pouch carcinogenesis of the Syrian golden hamster is probably the animal system process most comparable with the development of premalignancy and malignancy in human oral cancer. Furthermore, it is one of the most characterized models for squamous cell carcinomas (SCC). This was first developed by Salley (*1*), who produced an experimental oral SCC in the cheek pouch of the hamster. Later, Morris (*2*) standardized the procedures so that the experimental lesions could be uniformly reproduced. In the last 20 years, Shklar and coworkers have extensively studied the hamster cheek-pouch model and have performed a variety of chemoprevention experiments in this system.

The major advantages of the system as pointed out by Shklar et al (*3*) are the similarity between the hamster buccal pouch mucosa and the keratinizing human oral mucosa (in terms of histology, histochemistry, and ultrastructure), the absence of spontaneous carcinomas, the development of precancerous dysplastic lesions comparable to human oral leukoplakia, and the susceptibility of the tumor system to systemic influences, such as vitamins, hormones, and various drugs.

The carcinogenesis studies in the hamster cheek pouch are usually performed either by using multiple applications of 7,12-dimethylbenz(a)anthracene (DMBA) (usually 0.5% in mineral oil, three times weekly) or, occasionally, by using other carcinogens (*3,4*). The 0.5% dose of DMBA induced a hyperplastic response in the pouch epithelium after only a few applications, followed by the appearance of a variety of dysplastic lesions resembling human premalignant lesions after 6 to 8 weeks of treatment. Benign and malignant tumors (papillomas and SCC) started to develop after 10 weeks of treatment (*5*).

However, stages of carcinogenesis (initiation, promotion, and progression) have not been well defined in this system. To study the intimate cellular and molecular mechanisms involved in the genesis of oral tumors, a two-stage carcinogenesis animal model seems necessary in order to compare with the human oral cancer. The basic understanding of the pathogenesis of this tumor in the hamster cheek pouch is important for the development of new strategies of chemoprevention as well as for the study of chemopreventive agents and their possible mechanisms of action.

TWO-STAGE CARCINOGENESIS PROTOCOL

Tissue plasminogen activator (TPA) and benzoyl peroxide (BzPo) and other skin-tumor promoters produce well-defined, short-term effects on mouse skin, including sustained hyperplasia and proliferation. These short-term responses seem to be necessary events in the promotion process and are considered to be indicators of potential skin-tumor promotional activity of a compound (*6–9*). Although the phorbol ester is the most potent of the mouse-skin tumor promoters, it has been demonstrated that this compound has a very weak activity when applied in the hamster skin or cheek pouch (*10,11*). Furthermore, the lack of response of the cheek pouch to TPA has been confirmed in our laboratory (*12*).

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BzPo is a free radical-generating compound that has been demonstrated by Odukoya and Shklar to enhance the formation of tumors in the hamster cheek pouch when applied in combination with multiple treatment of DMBA (*13*). We analyzed a number of short-term markers of tumor promotion (keratins, hyperplasia, and nucleolar organizer regions [NORS]) in the hamster cheek pouch treated three times a week for 2 weeks with 40 mg of BzPo. Unlike in the mouse-skin model, BzPo was a more effective inducer of short-term markers of tumor promotion than was TPA (*12*).

CHEMOPREVENTION STUDIES

Chemoprevention has been studied in the hamster cheek pouch using a variety of natural and pharmacological agents. Many of these studies have focused on the use of vitamin A, vitamin E (alpha-tocopherol), and derivatives. Probably the most studied chemopreventive agent in the hamster cheek pouch has been vitamin E. Shklar showed that vitamin E significantly decreased tumor formation after 14 to 16 weeks of DMBA treatment; there were fewer tumors, and the tumors were smaller in size in the animals that received 10 mg of vitamin E twice weekly (*14–16*). Even more interesting were the experiments by Trickler and Shklar (*17*) using a lower dose of DMBA. Using 0.1% of DMBA instead of 0.5%, they found that tumor formation was completely inhibited by vitamin E.

The same group also studied the effect of vitamin E in established lesions of hamster cheek pouch (*18*). Hamsters that had been treated with DMBA for 13 weeks and developed SCC were then treated twice weekly for 4 weeks with 250 μg of vitamin E. In this study vitamin E was injected directly into the tumor-bearing pouch, and the treated hamsters were found to have a significant reduction in tumor mass. Microscopic examination showed that tumors were small and presented areas of degeneration with picnosis and inflammatory infiltration. Similar degenerative changes were also observed in leukoplakia.

The mechanism of the chemopreventive or the therapeutic action of vitamin E was not clearly established in these studies, but a study by Schwartz et al (*19*) showed that vitamin E prevented the depletion of Langerhans' cells caused by DMBA, suggesting an immunomodulating mechanism to explain, at least, the chemopreventive action of this compound.

Vitamin A and derivatives have also been extensively used in the hamster cheek-pouch model. A chemopreventive action of 13-*cis*-retinoic acid (10 mg given orally, twice weekly) was observed in animals treated with the standard DMBA protocol. Under this condition, the vitamin A derivative delayed tumorigenesis and reduced the size and number of SCC (*20*). Alteration in the distribution of Langerhans' cells in the epithelium and an alteration of the phytohemagglutinin blastogenesis was also observed, suggesting that the chemopreventive action of 13-*cis*-retinoic acid may also be mediated by the immune system (*21,22*).

One of the natural forms of vitamin A, beta-carotene, was also used in this system. In these experiments, in addition to the standard complete carcinogenesis protocol, a two-stage protocol using DMBA as the initiator and BzPo as the promoter was employed. Beta-carotene was shown to inhibit carcinogenesis in both protocols. In the two-stage protocol, beta-carotene appeared to act at both the initiation and promotion stages of carcinogenesis (*23*). Beta-carotene was also found to reduce the size and number of 1-glutamyl transpeptidase (GGT)-positive foci in DMBA-treated hamsters (*24*). Later, Schwartz and Shklar (*25*) suggested that the effect of beta-carotene may not be mediated by the metabolic conversion of this product to retinoid, the biologically active form of vitamin A. In this study, they used canthaxanthin, which is a carotenoid that is not converted into vitamin A. In the hamster cheek pouch, canthaxanthin was a potent antitumor agent, although not as potent as beta-carotene.

Chemoprevention by natural extracts was also investigated in this system. The extract of Spirulina-Dunaliella algae was shown to be a potent tumor inhibitor when given either alone (*26*) or in combination with beta-carotene. These compounds were also effective in producing regression of established tumors in the hamster cheek pouch (*27*).

Onion extract, administered orally in the drinking water, was also an effective inhibitor of DMBA tumorigenesis. This extract reduced cell proliferation in a cell line derived from a DMBA-induced SCC (*28,29*).

Many other compounds have also been investigated as chemopreventive agents in the hamster cheek pouch. Inhibitors of prostaglandin synthesis such as aspirin, indomethacin, and ibuprofen (*30,31*) have been shown to exert a significant antitumorigenic effect when given daily by mouth concomitant with the cheek-pouch DMBA topical treatment. The effect of two protease inhibitors, Bowman-Birk inhibitor (BBI) and soybean trypsin inhibitor (SBTI), was also examined in this chemically induced oral carcinogenesis model (*32*). The soybean extract containing the protease inhibitor BBI suppressed DMBA-induced carcinogenesis, whereas SBTI did not. Immunoenhancing agents such as bacillus Calmette-Guérin (BCG) and levamisole were found to inhibit the development of hamster buccal pouch tumor (*33–35*). The delay in carcinogenesis was observed clinically and histologically. Phenanthrene (Phe) and, to a lesser degree, 1,4-dimethylnaphtalene (DMeN) were found to retard the development of carcinomas when applied topically in the DMBA-treated hamster cheek pouch (*36*).

GGT AND KERATIN STUDIES

Although the mechanism(s) by which chemical carcinogens induce cancer in this system are not clearly defined, there have been some biochemical and molecular studies described. Probably the best-studied event has been the induction of GGT, an enzyme that is not normally expressed in the hamster cheek pouch. Solt and Shklar (*37, 38*) showed that individual positive GGT cells or doublet

cells are detected histochemically as early as 3 days after the first DMBA treatment. After 3 weeks of treatment, they were able to detect GGT-positive intraepithelial cell foci (plaques) that appeared to be of clonal origin. GGT activity has also been demonstrated histochemically in areas of dysplasia, papillomas, and well-differentiated SCC. Odajima et al (*39*) have speculated from these results that probably the early GGT-stained cell populations are preneoplastic in nature.

The expression of different keratins has also been explored in this model. A profile of several keratins during experimentally induced carcinogenesis in hamster cheek-pouch mucosa was studied by immunohistochemical techniques (*40,41*). The antibodies used in these experiments were capable of identifying several groups of keratins, but they were unable to recognize individual members of this family of proteins.

We have explored the expression of keratins using immunostaining with monospecific antibodies and also by using a technique that allowed immunoblotting analysis of tissues embedded in paraffin (*42*). Monospecific antibodies against murine keratins were developed by Roop et al (*43,44*) using synthetic peptides corresponding to the DNA sequence of the 3′ nonconserved region of keratin cDNA. These antibodies recognize single members of the keratin family and can be used to investigate the presence or absence of specific keratins in histological sections. We have used antibodies against three keratins: K14, which is normally associated with proliferating cells (basal layer); K1, which is normally expressed in differentiated cells of the epidermis; and K13, which is expressed in differentiated cells of mucosa. The pattern of keratins for the hamster cheek pouch was consistent with that of the oral mucosa (*45*).

K14 was restricted to the basal layer, K13 to the suprabasal layer, and there was no demonstrable immunoreactivity with K1 by either immunohistochemistry or immunoblotting. However, K1 was expressed by the cheek-pouch epithelium in a time-dependent fashion in DMBA-treated hamsters. Concomitant with DMBA-induced hyperplasia, there were some topographical alterations in the distribution of K14. In this case, K14 was no longer restricted to the basal layer but was also expressed in differentiated cells. The same pattern was also observed in dysplastic lesions and in squamous cell carcinoma. Furthermore, expression of the K13 differentiation-associated keratin was preserved in this hyperplastic epithelium during all the stages of carcinogenesis, including either anaplastic or differentiated areas.

Alteration in the pattern of keratin expression appeared to be a common feature in the development of SCC in different systems (*44–50*). These alterations probably reflect abnormal differentiation patterns and are excellent tools with which to monitor the process of carcinogenesis.

ONCOGENES AND SUPPRESSOR GENES

The level of expression of several cellular proto-oncogenes was examined at different stages of DMBA-induced tumor development in this model (*51*). This study demonstrated overexpression of c-Ha-ras gene at a very early stage of tumor development. Conversely, expression of c-erbB was detected after 8 to 10 weeks of DMBA treatment and increased with the progression of the disease. Expression of c-myc and c-sis detected in control tissues remained unaffected, while c-fos gene activity could not be detected at any stage of tumor development. It has been suggested that the increased expression of the ras gene can be correlated with the initial transformation activity of the hamster cheek-pouch epithelial cells, whereas activation of the c-erbB gene can be correlated with the extensive proliferative and malignant phenotype of these cells in the intact animal.

Recent studies have indicated that the c-erbB proto-oncogene and the transforming growth factor (TGF-α) may be involved in the mechanism of chemical carcinogenesis in this system. The c-erbB gene that is the cellular gene for the epidermal growth factor (EGF) receptor has been found to be overexpressed in DMBA-treated pouch epithelium and in cheek-pouch tumors (*52,53*). Furthermore, this gene appears to be amplified in cell lines derived from SCC (*54*). TGF-α, but not EGF, was also expressed in the pouch tumors, suggesting a possible autocrine stimulation mechanism involving TGF-α and the EGF receptor.

The same authors have demonstrated that c-Ki-ras mRNA can be detected in DMBA-induced tumors, whereas no detectable c-Ki-ras mRNA can be found in the normal cheek pouch. The c-Ki-ras proto-oncogene has been found to be proliferation dependent, and Wong et al suggest that this proto-oncogene is quiescent in the normal cheek pouch, although its expression is associated with malignant transformation (*55*).

Previous experiments in other systems showed that DMBA-induced tumors presented a specific mutation in the codon 61 of the Ha-ras gene (*56,57*). Recently, our laboratory has investigated whether a similar mutation occurs in the hamster cheek-pouch SCC induced by DMBA complete carcinogenesis.

The normal sequence of a fragment of genomic DNA encompassing codon 61 of the Ha-ras gene was amplified by the polymerase chain reaction (PCR) using primers designed for a highly conserved region of the mouse Ha-ras-1 gene. The sequence of the amplified fragment was determined by direct sequencing technique and exhibited 83.3% and 87.5% homology with the corresponding human and mouse sequences, respectively. Homology at the amino acid level was identical for the three species. Paraffin sections of 11 squamous cell carcinomas of the cheek pouch were used to detect mutated Ha-ras alleles. DNA sequencing of the tumors showed that 10 of 11 tumors presented, and A→T transversion in the second position of codon 61 resulting in amino acid change from glycine to leucine (*58*).

Moroco et al (*59*) have postulated the existence of three suppressor functions in this model, which strongly suggests that inactivation of suppressor genes may be involved during the process of carcinogenesis. These results

raise the possibility of a cooperation between an activated ras gene and inactivation of a suppressor gene for the progression to malignancy, as was also recently proposed for the mouse-skin carcinogenesis model (*60,61*).

OTHER STUDIES

A number of biological markers was studied at different stages of tumor development in this model in order to define intermediate end points for assessing the effects of chemopreventive or therapeutic agents (*62*). EGF receptor had been shown to be expressed in the hamster normal cheek-pouch epithelium but was moderately present in the hyperplastic epithelium and strongly expressed in both dysplasia and SCC, whereas transglutaminase I, polyamine levels, ornithine decarboxylase activity, and micronucleated cells were increased during all the stages of carcinogenesis.

Changes of NORs in the hamster cheek-pouch chemical carcinogenesis had been demonstrated using a silver colloid technique (*63*). This technique provides information on the nucleolar activity of the cell (rDNA transcription) and has been considered as a potential marker of malignancy (*64–66*). The number and degree of activity of NORs were determined in DMBA-exposed epithelium and the resulting tumors. The percentage of all types of NORs presenting high-activity nucleoli increased during DMBA treatment, reaching the highest values in the SCC.

CONCLUSIONS

Induction of tumors in the hamster cheek pouch is a well-characterized model of chemical carcinogenesis that has been extensively used in studies of chemoprevention and chemointervention. In the last few years, several biological markers have been characterized in this model. These markers can be used to understand mechanisms of carcinogenesis and, in addition, can serve as alternative shorter endpoints in studies of chemoprevention. In the next few years, the hamster cheek-pouch carcinogenesis model is likely to provide new clues into the intimate mechanism of cancer development. Some molecular studies have already been performed in this system with promising results, and it is expected that in the near future, with the use of new technology, it will be possible to define precisely the molecular events related to the different stages of tumor development. This information will be instrumental in understanding human oral cancer and in developing strategies for cancer prevention and treatment.

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Expression of Surfactant-Associated Protein in Non-Small-Cell Lung Cancer: A Discriminant Between Biologic Subsets

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ABSTRACT—We examined expression of the major surfactant-associated protein SP-A, a product characteristic of type II pneumocytes, in a panel of 126 non-small-cell lung carcinomas (NSCLC) by immunohistochemistry using routine paraffin-embedded material. In nonneoplastic lung, the expression was seen in normal and reactive type II pneumocytes. Based on a defined staining index composed of the number of cells and the staining intensity, 32% of all the NSCLC revealed SP-A immunoreactivity that was mostly focal. The highest incidence (50%) was demonstrated in adenocarcinomas having papillolepidic growth patterns (which include the bronchioloalveolar and papillary subtypes), followed by other subtypes of adenocarcinomas (31%) and other types of NSCLC (14%). Patients with SP-A expressing tumors had male/female ratio 1.1, as compared to 2.2 in all NSCLC or 2.0 in papillolepidic tumors. SP-A immunoreactivity in tumors was associated with lighter smoking history, significantly so in men (P_2 = .016). The finding of a marker characteristic of peripheral, alveolar cell differentiation in all adenocarcinomas is of interest and supports the concept of common pathogenesis for adenocarcinomas. We conclude that SP-A expression occurs predominantly, but not exclusively, in adenocarcinomas, particularly in those with papillolepidic growth pattern. SP-A expression may delineate biologically and clinically interesting subsets of NSCLC. [J Natl Cancer Inst Monogr 13:61–66, 1992]

Lung cancer has become the leading cause of cancer deaths in both men and women in the United States, with over 160 000 new cases diagnosed each year (*1*). Without a clear epidemiologic cause, the histopathology of lung cancer is changing (*2,3*), including a relative and absolute increase in the incidence of adenocarcinoma in the United States (*4,5*). The World Health Organization classification of lung cancers divides adenocarcinomas according to their predominant growth pattern into four subtypes: acinar (gland forming), solid with mucin production, papillary, and bronchioloalveolar (*6*). In our experience, up to 50% of all adenocarcinomas may demonstrate marked papillary or bronchioloalveolar growth patterns (*2*). Both of these subtypes have overlapping pathologic features and common histogenesis, and we refer to them collectively as papillolepidic tumors, after their main histopathologic features.

Most of the papillolepidic adenocarcinomas are tumors arising in peripheral airways, and their cellular origins include metaplastic mucin-secreting cells, Clara cells, and type II pneumocytes (*7,8*). Type II pneumocytes are the progenitor cells for the alveolar epithelium (*9,10*). Their major function is the production of surfactant, which exerts a detergentlike action essential for maintaining the patency of the alveoli, and they contain characteristic multilamellar secretory granules that represent the storage site of surfactant (*11,12*). Phospholipids make up the major component of surfactant, but several functionally important surfactant-associated proteins have been identified. The most abundant of these is surfactant protein SP-A (previously called SAP-35), which is a Mr 28 000–36 000 glycoprotein, also present in alveolar lavage (*13,14*). Specific antibodies against surfactant-associated proteins are available (*14–20*). Using a panel of lung cancer cell lines, we have previously reported that up to 41% (7/17) of adenocarcinoma and 83% (5/6) of the papillolepidic cell lines can express SP-A at the messenger RNA or protein level (*21*).

In this report we describe the expression of SP-A protein in a comprehensive panel of non-small-cell lung cancers (NSCLC) and the biographic data of the patients with these tumors. Most of the tumors expressing SP-A were adenocarcinomas, especially of the papillolepidic subtype. SP-A expression was also associated with distinct clinical features.

MATERIALS AND METHODS

We obtained 126 cases of NSCLC from the surgical pathology files of the Department of Pathology at

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[4] The opinions and assertions contained herein represent the private views of the authors and are not to be construed as official or as reflecting the views of the Department of the Navy or the Department of Defense.

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Bethesda Naval Hospital. Only well-fixed, surgically removed tumor material adequate for providing multiple serial sections for immunohistochemical and histochemical studies was used. Five-micron sections from routine formalin-fixed, paraffin-embedded blocks were cut and mounted on gelatin-coated slides.

Hematoxylin and eosin sections of each tumor along with mucicarmine staining were reviewed independently by two pathologists (R. I. Linnoila and A. F. Gazdar) in a blinded fashion. Consensus opinion was reached and tumors were classified according to the WHO criteria (*6*). Adenocarcinomas of the bronchioloalveolar and papillary subtypes were grouped together because both of these have papillolepidic growth characteristics and most of them probably arise from peripheral airways (*21*).

A rabbit SP-A antibody raised against a Mr 36 000 glycoprotein isolated from human amniotic fluid was a gift from Dr. S. N. Bhattacharyya, William Beaumont Army Medical Center, El Paso, Tex. The characterization of this antibody has been described elsewhere (*14*).

Immunohistochemical staining was performed by the avidin-biotinylated peroxidase-complex (ABC) technique using Vectastain ABC staining kits (Vector Laboratories, Burlingame, Calif.) according to the vendor's instructions, with previously reported modifications (*22*). Incubations with the primary antiserum were performed at 4 °C for 18 hours. A 1:1000 dilution was considered optimal because of strong staining in type II pneumocytes, whereas other pulmonary cells remained unstained.

The same positive control sections for the antibody against SP-A were included in each assay. Controls for the specificity of staining consisted of the incubation of serial lung sections with (1) nonimmune (normal) rabbit serum or (2) phosphate-buffered saline instead of the primary antiserum, resulting in omission of the immunoreactivity.

Results of the immunostaining were scored both for the number of positive tumor cells (distribution score: 0 = no positive cells; 1 for <10%; 2 for 10%–49%; 3 for 50%–100% of tumor cells positive) and for the intensity of the staining (0 = negative; 1 = weak; 2 = moderate; 3 = strong reaction). Using the sum of these values, an SP-A staining index (possible values: 0, 2–6) was established for each tumor (staining index = distribution score + intensity score; Tables 1–4; Fig. 3). A similar scoring system was used to evaluate mucicarmine histochemistry.

To study clinicopathologic correlations, 103 consecutive patients were included who were entered onto a therapeutic protocol of all stages of NSCLC at the National Cancer Institute dating from March 1984 to December 1987. The remaining 23 cases were chosen from surgical pathology files to establish optimal staining conditions and to be used as controls. All tumor specimens were obtained after appropriate informed consent, as required by the Institutional Review Board. We performed univariate analyses for the relationship between SP-A staining characteristics and the variables of histology, gender, and smoking history. Our initial analysis considered pack years of cigarette use grouped 0, 1–29, 30–39, 40–49, 50–69, 70–99, and 100 and over, but for purposes of presentation this was simplified as 0–49 and 50–500, as indicated. These analyses were performed using Fisher's exact test for 2 × 2 contingency tables or the Wilcoxon rank sum test. All *P*-values are two tailed and denoted P_2.

Table 1. The incidence of SP-A expression in various types of NSCLC

Histology	No. of tumors	SP-A staining index 3–6 (%)	SP-A >10% of tumor cells positive (%)
Adenocarcinoma	77	33 (43)*	16 (21)†
Papillolepidic	48	24 (50)‡	13 (27)§
Other	29	9 (31)	3 (10)
Epidermoid carcinoma	13	2 (15)	1 (8)
Large-cell carcinoma	26	1 (4)	0 (0)
Carcinoid	3	1 (33)	0 (0)
Adenosquamous	5	1 (20)	1 (20)
Other	2	2 (100)	0 (0)
Total	126	40 (32)	18 (14)

*P_2 = .001; adenocarcinoma vs. all other histologic types.
†P_2 = .013; adenocarcinoma vs. all other histologic types.
‡P_2 = .0012; papillolepidic subtype vs. all other histologic types.
§P_2 = .004; papillolepidic subtype vs. all other histologic types.

RESULTS

In nonneoplastic lung, moderate to strong staining was seen in solitary type II pneumocytes located in alveolar septae (Fig. 1,A). More intense immunoreactivity was detected in hyperplastic type II pneumocytes occurring in rows and in groups lining tumors, fibrotic septae, and pulmonary scars (Fig. 1,B). Staining was cytoplasmic, with nuclei being negative except for infrequently found minute positive nucleoli. Nonneoplastic cells with SP-A reactivity were also detected within tumors, regardless of the immunoreactive status of tumor cells (Fig. 2,A). These cells were smaller and not as pleomorphic as tumor cells. No staining was seen in the conducting airways. Alveolar macrophages showed variable reactivity.

The immunoreactivity for SP-A in tumors was mostly focal, detected in solitary or small groups of tumor cells (Fig. 2). Six out of 40 (15%) tumors with SP-A immunoreactivity demonstrated an intense and more diffuse staining pattern (≥50% of tumor cells positive; Fig. 2,C). All these tumors were adenocarcinomas; four of them were the papillolepidic subtype. Most of the tumors (80%) with SP-A immunoreactivity also expressed variable amounts of mucin (Fig. 3). Immunostaining was cytoplasmic, and the nuclei remained negative, except for nucleolar immunoreactivity, which was seen in 20 out of 40 (50%) tumors with SP-A immunoreactivity. The positive nucleoli in tumor cells were large and irregular in shape (Fig. 4).

The highest incidence (50%) of SP-A containing tumors was detected in the papillolepidic subtype of adenocar-

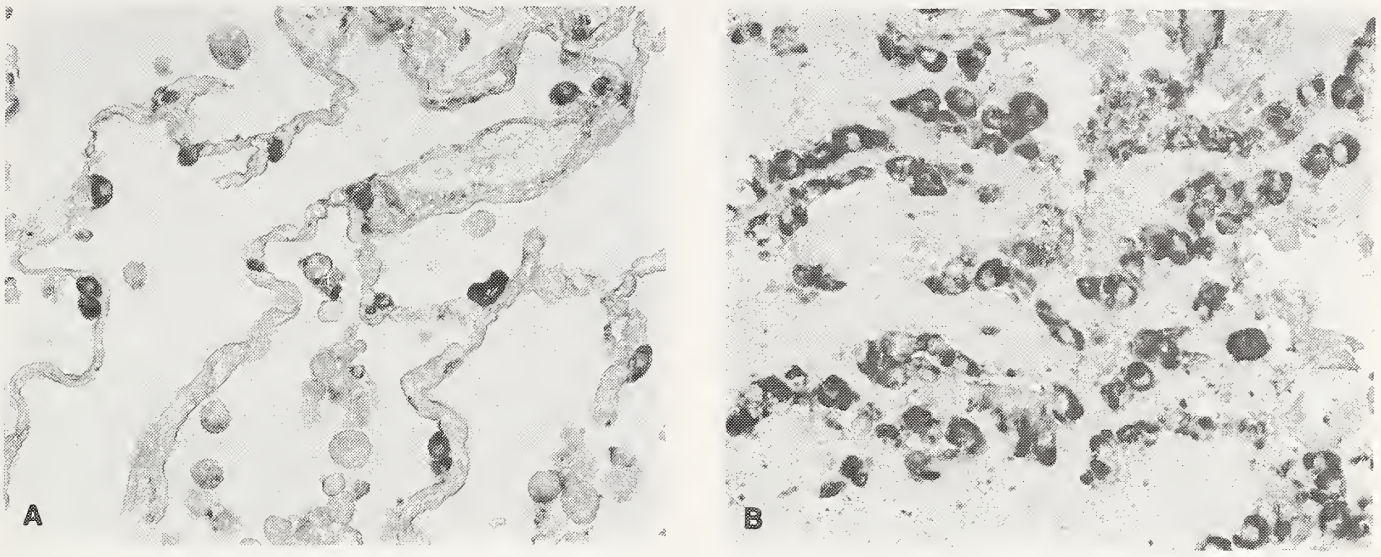

Fig. 1. Photomicrographs of SP-A immunoreactivity in nonneoplastic lung. (A) Normal alveoli with scattered solitary type II pneumocytes positive for SP-A (×360). (B) Thickened, fibrotic alveolar septae lined by rows and groups of reactive and hyperplastic type II cells positive for SP-A (×450).

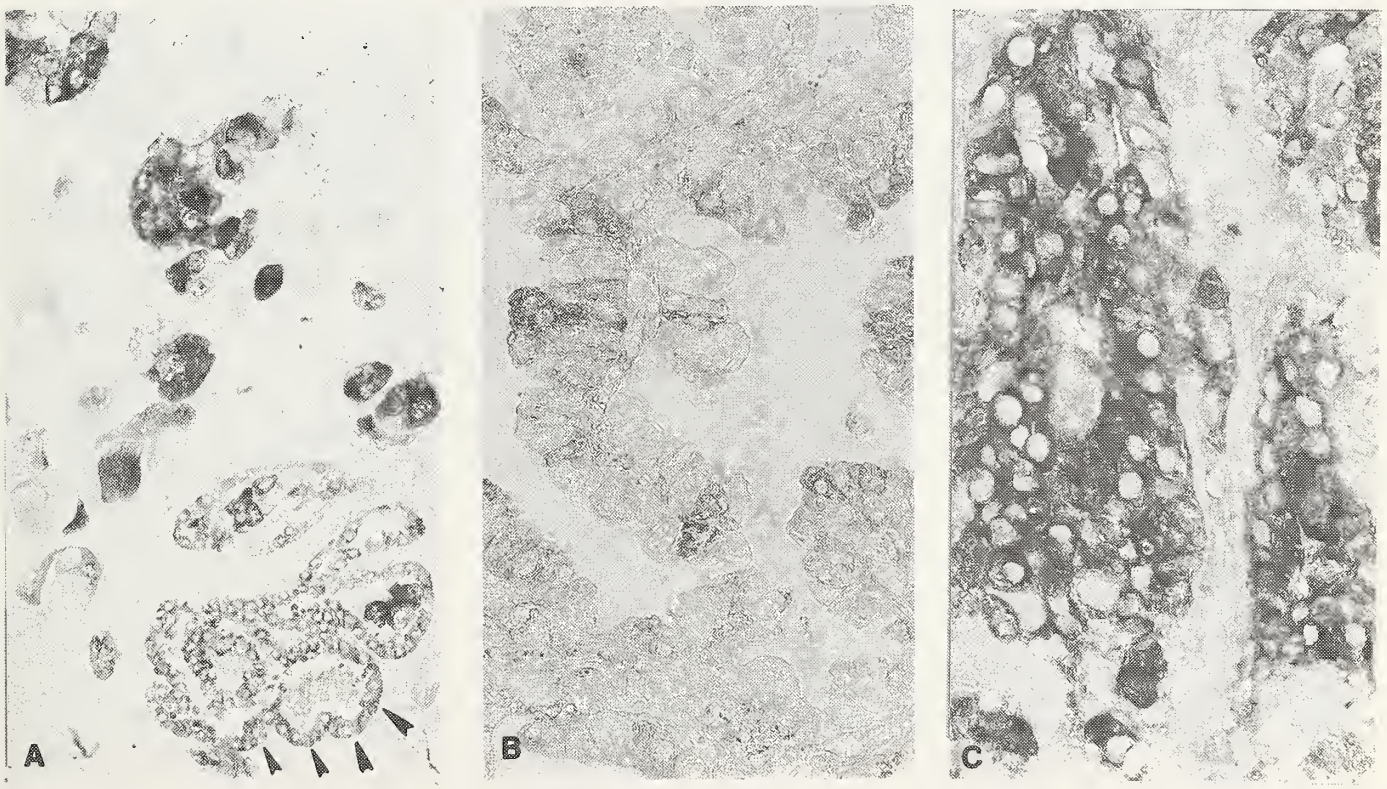

Fig. 2. Photomicrographs of characteristic patterns of SP-A immunoreactivity in tumors. (A) Scattered groups and solitary tumor cells with SP-A immunoreactivity in infiltrating papillary adenocarcinoma (larger cells). Note rows of smaller immunoreactive hyperplastic type II pneumocytes entrapped in the tumor (*arrowheads*, × 225). (B) A papillolepidic adenocarcinoma with variable intensity of SP-A staining (×360). (C) Intense diffuse staining in an acinar adenocarcinoma (×360).

cinomas, and 27% of the papillolepidic tumors had ≥ 10% of tumor cells positive for SP-A. Both incidences were significantly higher than in all other tumor types (P_2 = .0012 and .004, respectively; Table 1). Thirty-one percent of other adenocarcinomas and 14% of all other tumors showed expression of SP-A antigen.

Biographic data were available in 103 cases and are summarized in Table 2. The proportion of adenocarcinomas in males (54%) was not significantly different than the proportion (65%) in females (P_2 = .39), but the incidence of tumors with SP-A immunoreactivity was higher in women than in men (47% vs. 25%, P_2 = .04, Table 3). Specifically, among all the NSCLC and also in all the papillolepidic adenocarcinomas classified by routine light microscopy, the proportion of males was 67%; the proportion of males was 52% in the group of tumors with SP-A immunoreactivity (Fig. 5).

Because only a small fraction of the patients (7/103, 7%) was nonsmokers, we examined the relationship between SP-A immunoreactivity in tumors and smoking by grouping the patients according to pack years and gender. In this study, smoking habits between males and females were similar. Although there was a tendency for tumors with SP-A immunoreactivity to associate with lighter smoking history, this was statistically significant only in males (P_2 = .016; Table 4). It is of interest that five out of seven nonsmokers had SP-A negative tumors.

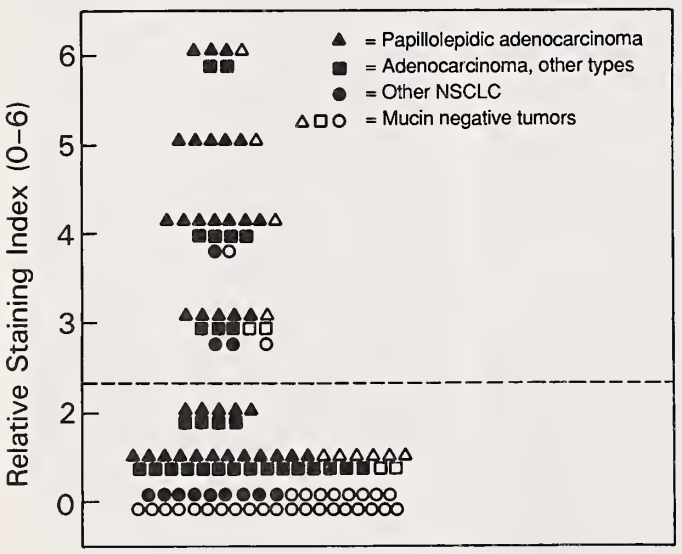

Fig. 3. SP-A immunoreactivity in NSCLC according to the staining index. Using the sum of distribution score and intensity score, a relative staining index was established for each individual tumor (n = 121; in 5/126 cases mucin stain was not available). Note that most (83%) of the tumors with high (5–6) staining indexes were papillolepidic adenocarcinomas. Most tumors (80%) with SP-A immunoreactivity were also positive for mucin (*solid symbols*). Tumors with staining indexes 3–6 (*above the dotted line*) were considered positive (Fig. 5).

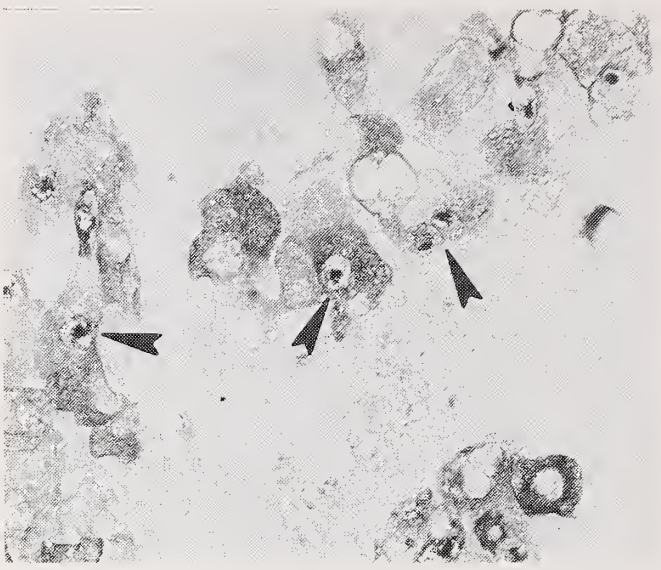

Fig. 4. Photomicrograph of SP-A immunoreactivity with nucleolar (*arrowheads*) and cytoplasmic staining patterns in a papillolepidic adenocarcinoma (×900).

Table 2. Patient biographic data

	SP-A positive*	SP-A negative†
Number of patients (n = 103)	33	70
Male gender (percentage)	17 (52)‡	52 (74)
Median age, years	57	56
Smoking history, mean pack years	43§	59

*Staining index 3–6.
†Staining index 0–2.
‡P_2 = .04; percent of males with SP-A positive vs. negative tumors (Fisher's exact test).
§P_2 = .034; pack years in SP-A positive vs. negative tumors (Wilcoxon rank sum test).

Table 3. Gender, histology, and SP-A immunoreactivity

	SP-A positive*	SP-A negative†
Male, total	17	52
Adenocarcinoma	16‡	21
Other NSCLC	1	31
Female, total	16	18
Adenocarcinoma	11§	11
Other NSCLC	5	7

*Staining index 3–6.
†Staining index 0–2.
‡P_2 = .0001; males with SP-A positive adenocarcinomas vs. other NSCLC.
§P_2 = .92; females with SP-A positive adenocarcinomas vs. other NSCLC.

DISCUSSION

The specificity of SP-A as a marker for type II pneumocyte differentiation was confirmed by its charac-

teristic staining pattern in the nonneoplastic lung. The antibody against SP-A gave strong reactivity in type II pneumocytes, whereas all other pulmonary epithelial cells, including type I pneumocytes and bronchial and bronchiolar epithelial cells, remained negative, which is in accordance with previous reports using other SP-A antibodies (*16,23,24*). Intense staining was also demonstrated by reactive, hyperplastic type II pneumocytes, a feature that is potentially helpful in distinguishing hyperplasias from carcinomas that often showed more focal or less intense immunoreactivity. Further studies are needed to establish whether patterns of SP-A expression may also be useful as an early indication for carcinogenic changes of peripheral airway and alveolar cells.

Our results indicate that papillolepidic growth pattern is the predominant, but not exclusive, growth pattern of adenocarcinomas with SP-A expression. This is in accordance with our previous findings on cell lines (*21*). The SP-A immunostaining was focal in most cases, suggesting clonal expansion of subpopulations of tumor cells and confirming the heterogenous cellular composition of adenocarcinomas. Similar patterns were detected using RNA-RNA in situ hybridization for the expression of SP-A mRNA (*25*).

The incidence of tumors with SP-A immunoreactivity in our paper is in accordance with previous immunohistochemical reports (*16,17,23,26*). However, the incidence is higher than expected on the basis of ultrastructural studies (*27*). It is conceivable that neoplastic type II pneumocytes may express SP-A but have lost the ability to form multilamellar bodies, the ultrastructural characteristic of type II cell differentiation. In addition, malignant transformation in NSCLC tumor cells may be

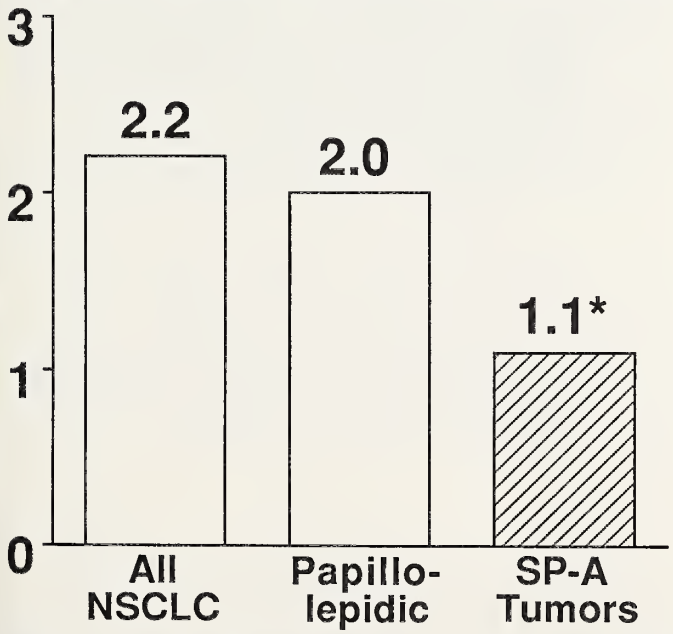

Fig. 5. Decreased male/female ratio in SP-A positive tumors. The male/female ratio in SP-A positive tumors (n = 33, staining indexes 3-6, *hatched bar*) was lower (*asterisk*, P_2 = .04 by Fisher's exact test) than in all papillolepidic tumors that were diagnosed by routine light microscopy using only hematoxylin and eosin stain.

Table 4. Smoking, gender, and SP-A immunoreactivity

	Pack years	
	0–49	50–500
Males		
SP-A positive*	13‡	4
SP-A negative†	20	31
Females		
SP-A positive*	11§	5
SP-A negative†	10	8

*Staining index 3–6.
†Staining index 0–2.
‡P_2 = .016; SP-A positive tumors in males with 0–49 vs. 50–500 pack years (Fisher's exact test).
§P_2 = .66; SP-A positive tumors in females with 0–49 vs. 50–500 pack years (Fisher's exact test).

associated with inappropriate production of SP-A, which is not limited to tumors arising from type II pneumocytes. These findings are further supportive evidence that all adenocarcinomas share a common histogenetic origin.

Our study provides new information on the clinical features of tumors with SP-A expression. Tumors with SP-A immunoreactivity were found more frequently in women. It is interesting to speculate whether this might be due to hormonal factors. Hormones such as prolactin and corticosteroids are known to increase SP-A synthesis in selected conditions (*11*). Yet papillolepidic growth pattern in our study was not associated with female gender. Moreover, tumors with SP-A immunoreactivity were seen more frequently in patients with a lighter smoking history. Our results suggest that smoking is associated with the loss of a highly differentiated function, the production of SP-A in tumors. A major concern arising from this analysis is that other, as yet unidentified, factors than smoking may play a role in the pathogenesis of human NSCLC, particularly of adenocarcinomas. These concerns were also raised by a recent study on the changing pattern of lung cancer in Japan (*28*).

In conclusion, SP-A expression is predominantly but not exclusively seen in adenocarcinomas, especially with papillolepidic growth patterns. Applications of SP-A include identification of potential premalignant changes of adenocarcinomas as well as biologically and clinically distinct subsets of NSCLC. Although we have confirmed and extended our current findings in a study including multiple peripheral airway cell differentiation markers (*29*), further analysis of SP-A expression in other, independent and larger patient populations are needed to confirm our observations on smoking history and lung cancer type.

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Tumor Antigen Phenotype, Biologic Staging, and Prognosis in Head and Neck Squamous Carcinoma[1]

Gregory T. Wolf, Thomas E. Carey[2,3]

ABSTRACT—Prior studies of alterations in tumor expression of normal blood group antigens and A9/$\alpha^6\beta_4$ integrin, an extracellular matrix receptor, have suggested that these immunohistologic markers reflect the biologic aggressiveness of head and neck squamous carcinomas. To confirm these preliminary observations, prospective long-term follow-up of 82 previously untreated head and neck squamous carcinoma patients was performed. All patients were treated with conventional therapy. Median follow-up was 57 months. Tumor immunohistology for ABH blood group and A9/$\alpha^6\beta_4$ integrin expression was performed and correlated with measures of host cellular immunity, disease-free survival, and overall survival. Loss of blood group expression and high A9/$\alpha^6\beta_4$ integrin expression were each directly related to an increased frequency of early tumor recurrence. The combination of both variables was significantly associated with both disease-free (P = .029) and overall survival (P = .05). Increased expression of A9/$\alpha^6\beta_4$ was associated with impaired T-lymphocyte function (P = .005), and loss of blood group expression was associated with decreased peripheral blood levels of $CD8^+$ T-lymphocytes (P = .013). The findings suggest that these phenotypic characteristics of antigen expression in head and neck squamous carcinomas are important markers of biologically aggressive cancers and impaired host immune response. The clinical use of these biologic staging parameters in the initial assessment of patients should allow selection of more aggressive primary treatment strategies for individual patients. [J Natl Cancer Inst Monogr 13:67–74, 1992]

Conventional treatment strategies for squamous carcinomas of the head and neck consist of surgical resection or radiation therapy for patients with early cancers or a combination of these treatments for patients with advanced cancers. Despite the effectiveness of surgery and radiation therapy for limited cancers, 10%–30% of patients will experience tumor recurrence or metastases, and 20%–40% will develop second primary carcinomas, particularly in the head and neck region. Cure rates for more advanced cancers are poor, ranging from 0% to 40%, and most treatments are associated with severe functional and cosmetic disabilities.

Current clinical staging parameters have not been adequate to define those high-risk individuals with early disease who will suffer relapse after conventional therapy or those individuals with advanced disease who might be cured with either radiation or surgery alone. Because of this, recent interest has turned toward more precise markers of the biologic aggressiveness of these cancers, using both histologic and immunologic techniques. Of particular interest are studies of tumor-cell antigen phenotype (*1–4*). Recent studies of the expression of the A9/$\alpha^6\beta_4$ integrin and the ABH blood group antigens suggest that these markers may be useful in identifying patients likely to suffer early tumor relapse and death (*5–7*). We have now studied and followed a prospective cohort of treated patients using these parameters to determine their value as prognostic factors. Final results of long-term follow-up are reported here.

METHODS

Patient Population

Eighty-two patients with biopsy-proven, previously untreated head and neck squamous carcinoma were studied prospectively. Twelve patients had early disease (stage I or II), and 68 patients had advanced disease (stage III or IV). Stage was not specified in two patients. The oral cavity was the primary site in 27 patients, the oropharynx in 16 patients, the larynx in 27 patients, the hypopharynx in 11 patients, and the external ear in one patient. Patient age ranged from 29 to 87 years (median, 59 years). All but two patients were males. All patients underwent radical surgical resection for cure. Postoperative radiation therapy was added in those patients with stage III or IV tumors. Follow-up ranged from 2 to 93 months. Median follow-up was 57 months. All patients were included in analysis except for two who were lost to follow-up and were excluded from survival analysis.

Tumor Phenotype

At the time of surgery, representative, nonnecrotic tumor samples (1 cm^3) were obtained from each resected tumor specimen at the tumor-normal tissue interface. The

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specimens were quickly frozen in OCT (Tissue-Tek, Miles, Inc.). Serial 5-μm frozen sections were fixed in cold acetone for 20 seconds and stored frozen prior to hematoxylin and eosin (H-E) and immunohistologic staining. The sections were stained using commercially prepared monoclonal antibodies to blood groups A and B (Accurate Chemical and Scientific, Westbury, N.Y.) and the UM-A9 and UM-G10 antibodies developed in our laboratory (*8*, *9*). Antibody binding was detected using the Vectastain kit from Vector Laboratories according to kit instructions. Sequential cryostat sections were used in each experiment. The first and last sections were stained with H-E to confirm by conventional histologic criteria the presence and location of tumor within each tissue specimen. The properties of monoclonal antibodies UM-A9 and UM-G10 have been described elsewhere (*8,9*). These antibodies were raised in the same fusion from splenocytes of a mouse immunized with the human squamous carcinoma cell line UM-SCC-1. The antigen identified by UM-A9 is displayed at the basal pole of normal basal keratinocytes (*8*) and identifies a newly defined member ($\alpha^6\beta_4$) of the integrin superfamily of extracellular matrix receptors (*10*). The G10 antigen is the H type 2 blood group antigen (*9,11*) and was used as the reagent for the H blood group precursor (type O) antigen. Tissue sections were scored for intensity of staining and for reactivity of blood group antibodies and compared with endothelial cells and normal mucosal epithelium. This served as an internal control for A, B, and H expression. Adjacent nonmalignant mucosa for confirming blood group expression in normal epithelium was available in all but 14 cases. All patients were independently blood typed by the hospital laboratory. The expression of appropriate blood group antigens in tumor tissue was compared to that in the adjacent normal tissue and the patient's blood type and was classified as being present (retained) or absent (lost) in the tumor tissue. The expression of A9/$\alpha^6\beta_4$ antigen was characterized as one of three distinct patterns according to previously described criteria (*5–7*). Pattern 1, the strongest expression of A9, was characterized by dark outlining of the invading edge of tumor nodules and homogeneous intense staining of individual malignant cells. Pattern 2 was characterized by strong to moderately strong outlining of tumor nodules with weak or absent staining of cells away from the invading tumor border. Pattern 3 represented the weakest expression of A9 and was characterized by faint outlining of tumor nests and patchy staining of individual cells, with many cells not stained. A few tumors exhibited areas of differing staining pattern and were scored according to the strongest pattern observed. Patients were grouped for analysis according to high expression (pattern 1) versus low expression (patterns 2 or 3) of A9/$\alpha^6\beta_4$. Slides were read prospectively by two investigators blinded as to patient outcome.

Host Immune Response

Immunologic parameters of the host cellular immunity were concomitantly assessed in 53 patients prior to treatment. Measured were in vitro production of the lymphokine, leukocyte migration inhibitory factor (LIF), and peripheral blood levels of CD4 and CD8 positive lymphocytes. Pretreatment blood samples were obtained from each patient at 8:00 AM on the morning of the assays. White blood cells were separated from the whole blood by gravity sedimentation and washed in RPMI-1640 medium (GIBCO, Grand Island, N.Y.), supplemented with 10% fetal bovine serum (medium). In vitro leukocyte migration inhibition (LMI) in response to phytohemagglutinin (PHA) was determined by use of a standardized agarose microdroplet technique (*12–14*). In brief, 2×10^7 viable cells were suspended in 0.1 mL of 0.2% agarose. Two-microliter agarose droplets were placed in the center of each well of a 96-well microculture plate with a 100-μL repeating syringe. Then, chilled (4 °C) medium-containing PHA (1 μL/mL) or an equal volume of diluent was added and, after an 18-hour incubation at 37 °C, areas of leukocyte migration were measured with an inverted microscope, ocular micrometer, and mechanical stage. Percent migration inhibition was calculated from the ratio of mean areas of quadruplicate migrations in medium alone, compared to medium with PHA. The results of this assay are a direct reflection of in vivo delayed hypersensitivity responses, with a normal response defined as a percent migration inhibition greater than 20% and an abnormal response as less than 20% (*13,14*).

For determination of lymphocyte subpopulations, heparinized whole blood (10 mL) was studied using flow cytometry and commercially available monoclonal antibodies for CD4 and CD8 lymphocytes according to previously described techniques (*14,15*). Briefly, leukocytes were stained with monoclonal antibodies by an indirect immunofluorescent technique in a whole-blood preparation. Whole blood (100 μL) was incubated on ice with 50 μL of unconjugated monoclonal antibody at a predetermined concentration for 15 minutes. The red blood cells were lysed by the addition of ammonium chloride solution, incubated at room temperature for 10 minutes, and washed twice in media. A fluorescenated second antibody (goat antimouse) was added, and after two washes, the labeled cells were resuspended in medium and analyzed on a multiparameter flow cytometer (Epics C, Coulter Corp.). The argon-ion laser was tuned to 488 nm at a power of 500 mW. The lymphocytes were separately analyzed from the monocytes and granulocytes by electronic gating with forward-angle light scatter and 90° light scatter. Histograms were analyzed with a software routine (IMMUNO) on a data analysis system (EASY). Percent positives were determined by comparison with negative control histograms. For analysis, patients were grouped according to whether they were above or below a level of CD8 lymphocytes that was previously determined to be 1 standard error below the mean for a normal control population (*15*). Likewise, grouping of patients according to CD4 levels was accomplished in the same fashion.

Statistics

The association of immunohistologic staining characteristics with tumor characteristics, immune response, and

frequency of relapse or death was determined by chi-square analysis. Comparisons of disease-free interval and determinant survival were performed between groups by life table survival analysis using log-rank methods. Comparisons of mean levels of immune response parameters among patients grouped by tumor antigen phenotype were made using Student's *t*-test and confirmed using nonparametric methods. All *P* values correspond to two-sided significance tests.

RESULTS

Disease-Free and Overall Survival

Of the entire group of 82 patients, 49 (60%) of these patients died of cancer, six (7%) died of other causes, 26 (32%) remained alive and disease free, and one patient was lost to follow-up. Tumor recurrence occurred in 51 patients (62%). Median determinant survival for the entire group was 25 months. Median length of follow-up was 57 months (range, 2–93 months).

Blood Group Expression

Loss of normal expression of ABH blood group antigens in tumor tissue was associated with relapse (P = .066). Of 25 patients with loss of expression, 19 recurred (76%), with a median disease-free interval of 8.1 months. Of 43 patients whose tumor tissue retained expression of appropriate blood group, 23 recurred (53%), with a disease-free interval of 32.3 months. Overall disease-free survival was significantly longer when appropriate blood group antigens were expressed by the tumor tissue (P = .046) (Fig. 1). Although overall determinant survival curves did not differ significantly according to blood group expression in the tumor, there was a trend (P = .171) for increased survival in patients with retained expression (median survival, 30.0 months) compared to patients with loss of expression (median survival, 16.8 months).

A9/$\alpha^6\beta_4$ Integrin Expression

Expression of the A9/$\alpha^6\beta_4$ integrin varied among the patients and was independent of blood group expression. Tumors in 48 patients showed high expression (pattern 1) compared to 34 patients whose tumors showed less intense patterns of immunohistologic staining (pattern 2 or 3). Long-term prospective follow-up of this cohort of patients with predominantly advanced disease showed that both disease-free interval and determinant survival duration were increased in patients with low A9/$\alpha^6\beta_4$ expression. Median disease-free interval in patients with low expression was three times that found for patients with high expression (P = .12) (Fig. 2). Median determinant sur-

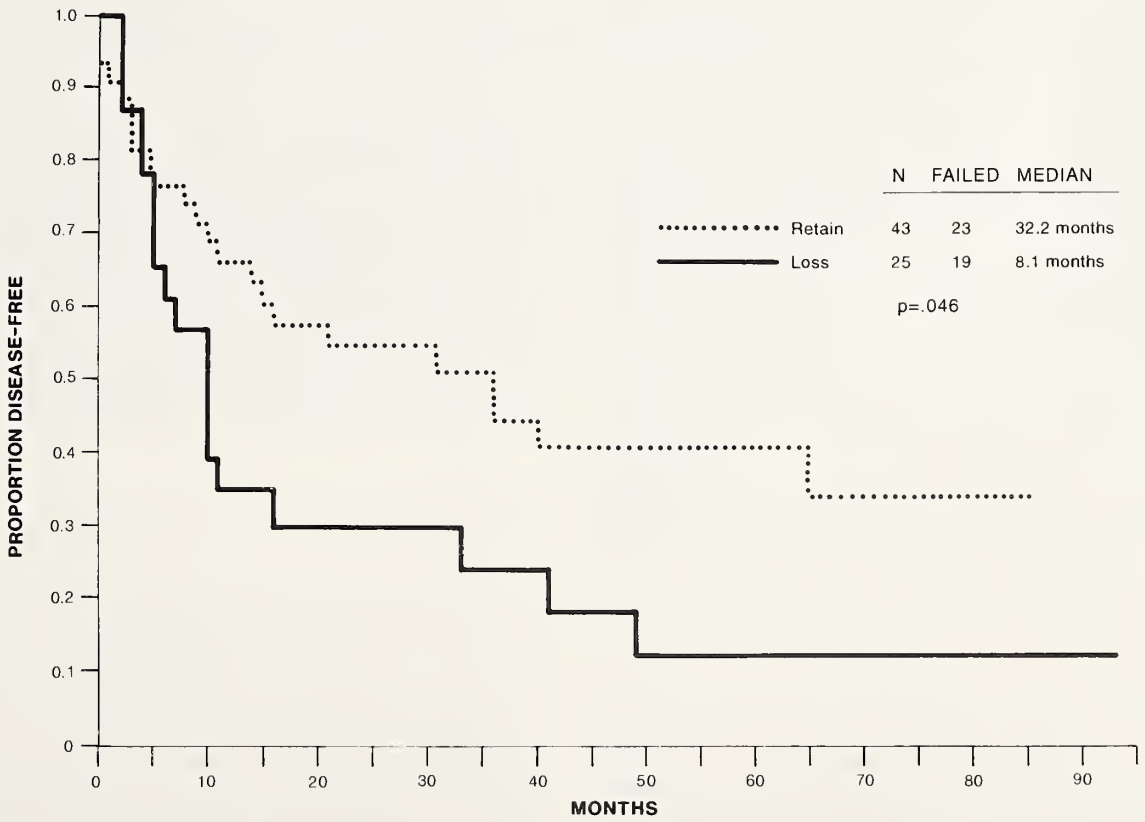

Fig. 1. Disease-free survival according to tumor expression of ABH blood group. Median disease-free interval was significantly longer in patients whose tumors retained blood group expression compared to those who had lost expression.

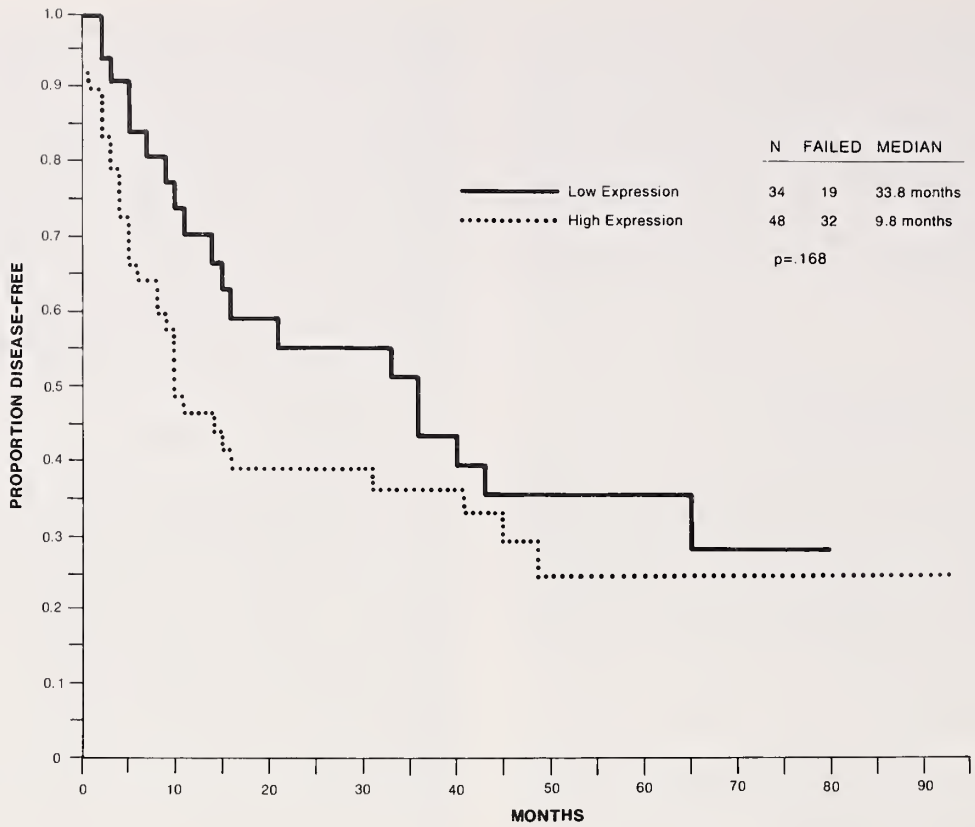

Fig. 2. Disease-free survival according to tumor expression of A9/$\alpha^6\beta_4$ integrin in patients with head and neck squamous carcinoma. High expression was associated with early tumor recurrence and shortened median disease-free interval.

vival durations were 33 months and 19 months, respectively.

Among patients whose tumors retained appropriate expression of blood group or in whom blood group expression was indeterminant, low expression of A9/$\alpha^6\beta_4$ was useful in identifying patients with the most favorable prognosis. Median disease-free interval was 38 months in this group of patients compared to 14.2 months in patients with high expression of A9/$\alpha^6\beta_4$ and normal or indeterminant blood group expression (P = .029) (Fig. 3). Likewise, overall determinant survival differed significantly when both blood group expression and expression of A9/$\alpha^6\beta_4$ were considered together (P = .05) (Fig. 4). Of 26 long-term survivors, only four had tumors with loss of blood group expression, and three of these four had tumors with high A9/$\alpha^6\beta_4$ expression.

Correlations of Immunohistology With Host Immune Status

In vitro lymphokine production by peripheral blood lymphocytes was determined prior to treatment in 53 patients. The ability of lymphocytes to respond appropriately to phytohemagglutinin was impaired in 29 patients and normal in 24 patients. Impaired response was significantly associated with high expression of the A9/$\alpha^6\beta_4$ integrin in the tumor (P = .005) (Fig. 5). Blood group expression, however, was not associated with in vitro lymphokine production.

The relationship of A9/$\alpha^6\beta_4$ and blood group expression to levels of CD4 and CD8 positive lymphocyte populations in the peripheral blood of the cancer patients was also assessed. The only significant relationship found was an association of loss of blood group expression with low levels of CD8 positive lymphocytes in the peripheral blood (P = .013). Further, mean CD8 levels were significantly lower (19.2 ± 6.3%) in this group than in patients whose tumors retained blood group expression (25.4 ± 7.7%) (P = .009).

DISCUSSION

The findings in our long-term study of tumor antigen expression suggest that alterations in the expression of specific normal antigenic constituents of the epithelium in malignant epithelial cells correlate with the biologic behavior of these cancers. Prognostically, loss of expression of normal blood group antigens appears to be more important than increased expression of the A9/$\alpha^6\beta_4$ integrin. Abnormal expression of blood group antigens in a variety of cancers has been previously reported (*4,16–19*). These studies have generally found that loss of expression correlates with tumor invasiveness or metastatic capability (*17*,

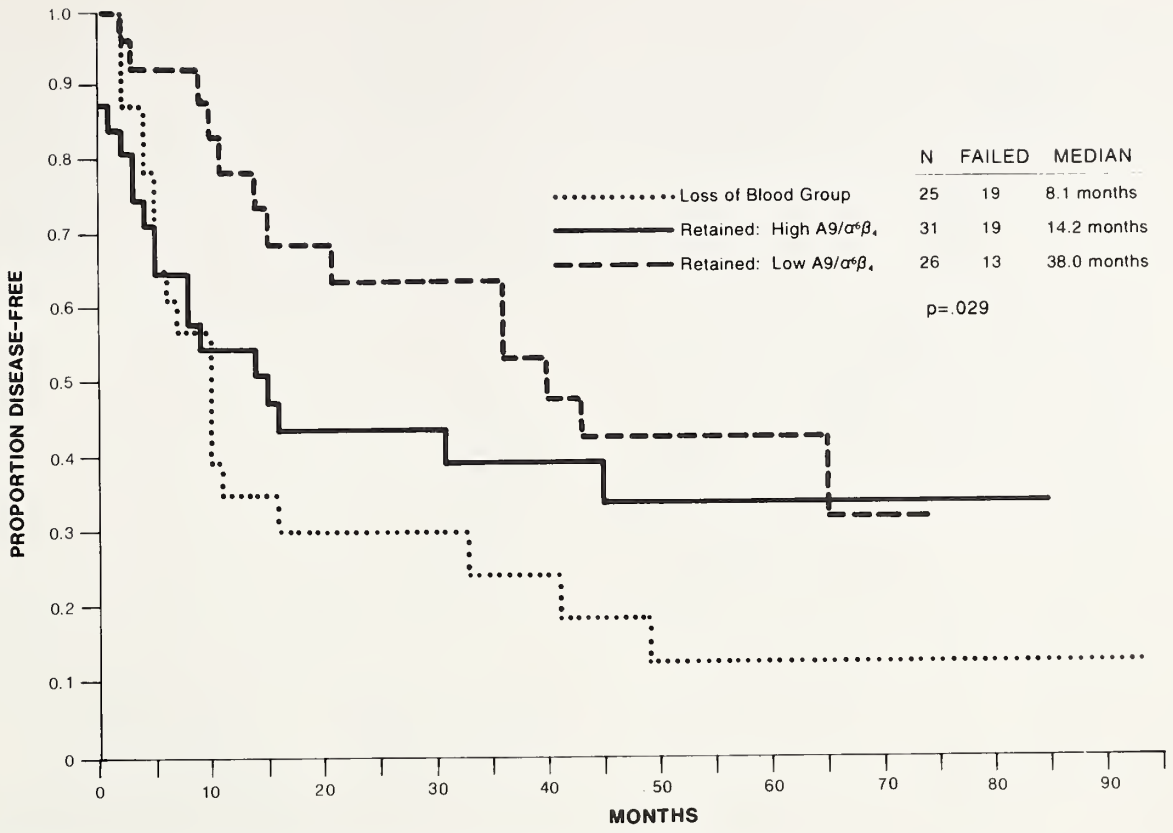

Fig. 3. Disease-free survival according to loss of blood group (ABH) expression and expression of the A9/$\alpha^6\beta_4$ integrin. The longest median survival was associated with tumors showing low A9/$\alpha^6\beta_4$ expression and retained blood group expression.

19). Our initial studies, which involved a small group of patients with limited follow-up, suggested that this immunohistologic finding was of prognostic importance in head and neck squamous carcinoma (*5,6*). The current study with extended follow-up of a larger group of patients confirms these preliminary observations and suggests that blood group expression may be a clinically useful biologic marker for squamous carcinomas. Previous Cox regression analysis comparing blood group expression to traditional prognostic factors has also supported the superiority of blood group expression in predicting early tumor relapse (*7*).

The molecular and genetic basis for loss of blood group expression in specific head and neck cancers remains speculative. It is most likely a reflection of altered tumor cell differentiation that results in changes in the molecular structure or branching of blood group glycolipids or changes in coding for transferases necessary for the building of carbohydrate chains (*20*).

The results of this study also suggest that increased expression of the A9/$\alpha^6\beta_4$ integrin is associated with clinically aggressive cancers and can provide additional prognostic information independent of blood group expression. Within the large subgroup of patients with tumors that expressed normal blood group antigens, the expression of A9/$\alpha^6\beta_4$ separated those patients into two groups with different clinical outcomes. The patterns of low expression of A9/$\alpha^6\beta_4$ (patterns 2 and 3), which were found to correlate with longer disease-free interval, are reminiscent of the staining patterns seen in normal epithelium in which expression is limited to the basement membrane and basal cells. In the cancers with low expression, antigen expression was limited to cells at the epithelial-stromal interface, with little or weak expression in the general population of tumor cells.

The A9/$\alpha^6\beta_4$ molecule appears to be a member of the integrin family of extracellular matrix receptors. Increased expression of this newly described integrin appears to correlate with the aggressiveness of tumor cell lines (*10*). Because the integrins are molecules related to extracellular matrix receptors, alterations in expression may have profound influences on tumor cell adhesion properties, metastatic capability, invasiveness, and the function of other growth-regulating cell surface receptors (*21–24*). Studies are currently underway to determine the relationship of A9/$\alpha^6\beta_4$ expression with tumor cell proliferation, aneuploidy, and tumor chemosensitivity in vivo.

An unexpected finding was the strong correlation of high A9/$\alpha^6\beta_4$ expression with impaired in vitro lymphocyte function in the cancer patients. This represents the first demonstration of a specific tumor antigenic alteration that was associated with a parameter of impaired host

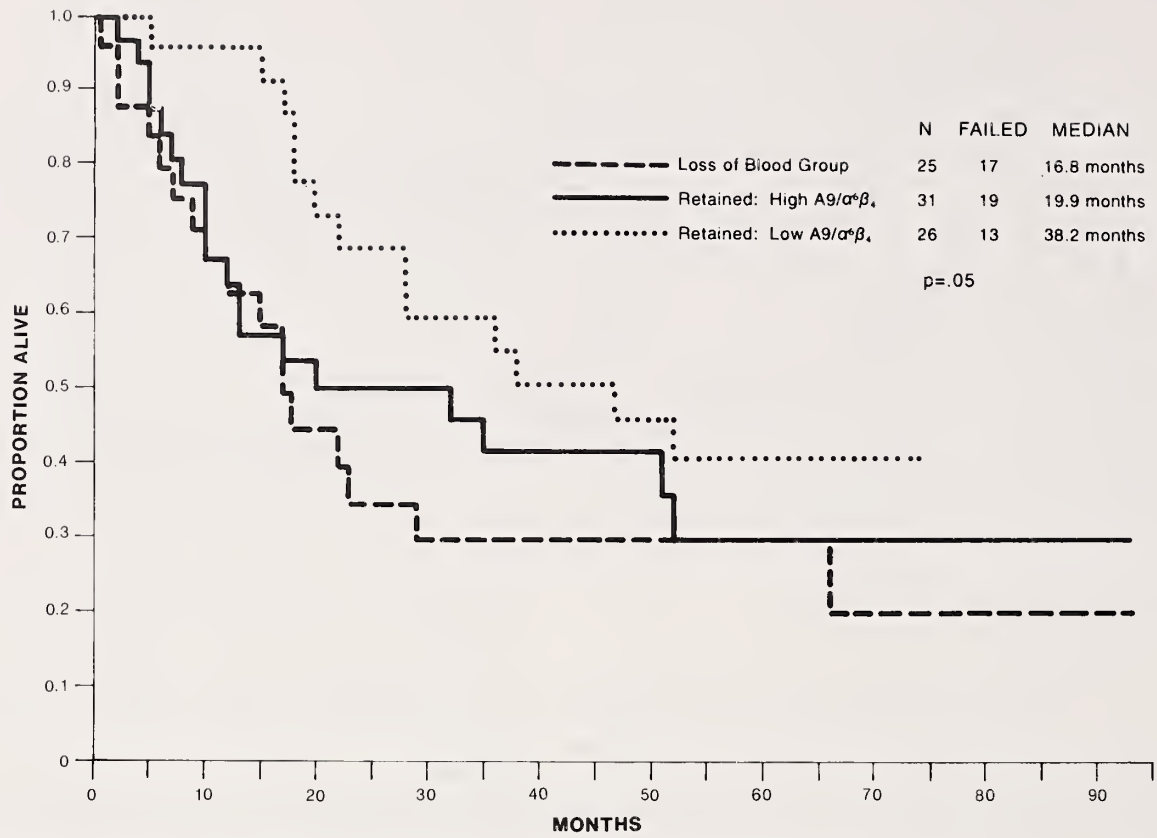

Fig. 4. Overall determinant survival according to expression of blood group (ABH) and A9/$\alpha^6\beta_4$ integrin.

cellular immunity. Impairments in T-lymphocyte numbers and function are well recognized in patients with advanced head and neck cancer (*14,15,25*). Understanding the basis for these immune abnormalities has been elusive; however, the impairments have been consistently correlated with tumor burden and with poorly characterized serum immunosuppressive factors (*26,27*). Some of these serum factors have been identified as circulating immune complexes (*28*), the levels of which have been related to both immune suppression and tumor response to chemotherapy (*29,30*). It has also been suggested that high levels of specific cell membrane receptors that are shed into the circulation, such as interleukin-2 receptors, are immune suppressive and correlate with poor prognosis (*31,32*). Whether extracellular matrix receptors such as A9/$\alpha^6\beta_4$ have similar effects remains unknown. High A9/$\alpha^6\beta_4$ expression was associated with impaired ability of T lymphocytes to respond appropriately to mitogenic stimulation and produce an indicator lymphokine (LIF). The coincidence of this antigenic characteristic of tumor aggressiveness and cellular immune impairment may explain in part the poor prognosis of some patients. Likewise, the association of loss of blood group expression in a tumor and low levels of peripheral blood CD8 cells may also reflect a poor prognosis (*15*). These observations and their potential interrelationship are unexplained associations that pose provocative questions for future studies.

At present, confirmation of these findings in larger prospective trials is needed. Currently, the correlation of tumor antigen phenotype with prognosis is being pursued through a Veterans Administration Cooperative Study of induction chemotherapy for advanced laryngeal cancer (*33*). However, studies of patients with earlier cancers and of those who develop second primary malignancies are also needed.

Future use of these biologic staging parameters as part of the initial assessment of patients with early head and neck cancers could allow selection of more aggressive primary treatment strategies for individual patients. In addition, ongoing studies in patients receiving induction chemotherapy should indicate the potential usefulness of these biologic staging parameters for predicting tumor response to chemotherapy. Such information would be valuable in planning adjuvant chemotherapy programs and in selecting patients for organ preservation treatment strategies that use induction chemotherapy.

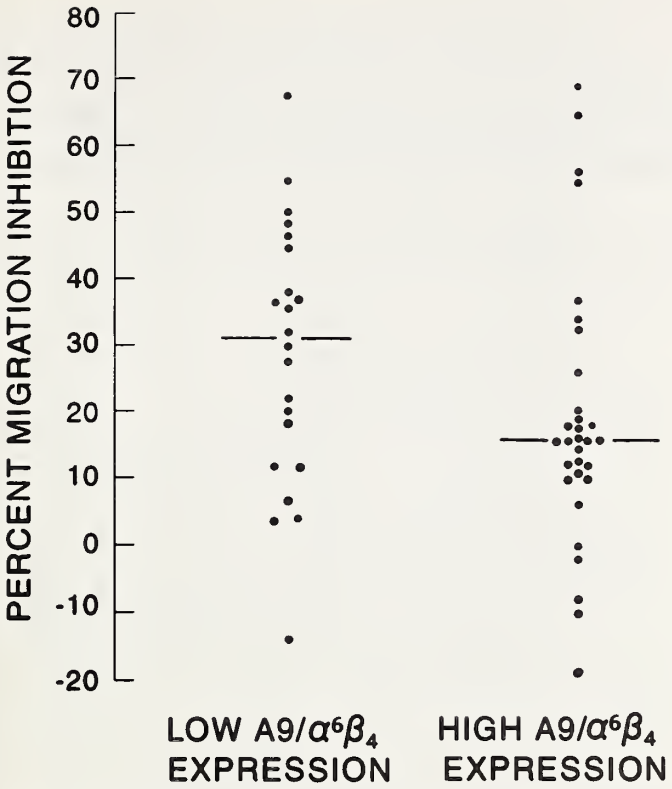

Fig. 5. Production of lymphokine (leukocyte migration inhibitory factor) in vitro by lymphocytes from patients with tumors showing high or low expression of A9/$\alpha^6\beta_4$ integrin. High expression was significantly associated with impaired lymphokine production (<20% migration inhibition) in 23 of 31 patients (74%); P = .005 (Pearson's chi-square). Median values indicated by line.

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Regulation of Expression and Phosphorylation of A9/$\alpha^6\beta_4$ Integrin in Normal and Neoplastic Keratinocytes[1]

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ABSTRACT—The A9 antigen is a basement membrane antigen of normal squamous epithelial cells that is strongly expressed in many squamous carcinomas. High expression of this antigen is associated with early relapse in squamous cell carcinomas of the head and neck. We now know that the A9 antigen is structurally, immunologically, and functionally similar to the $\alpha^6\beta_4$ integrin that has been shown to be linked to metastatic behavior in murine tumor models. The α^6 and β_4 genes have been cloned and sequenced, and a model has been constructed from the deduced amino acid composition. In this study we present a hypothetical model and use it to design experiments to assess the factors that influence the expression of the A9/$\alpha^6\beta_4$ integrin in normal and malignant keratinocytes. High calcium induces down regulation of A9/$\alpha^6\beta_4$ antigen in normal but not malignant keratinocytes within 24 hours. Although calcium can downregulate β_4 message in tumor cells in the absence of epidermal growth factor (EGF), transcription of β_4 increased in the tumor cells under the conditions we used for assessing antigen expression (calcium plus EGF). Retinoic acid also stimulated transcription of β_4 in tumor cells, but this was partially inhibited by the presence of high calcium. Phosphorylation of the β_4 chain was stimulated by epidermal growth factor and calcium in normal keratinocytes, but in the malignant cells phosphorylation was constant regardless of the culture conditions. Our results indicate that high expression of the $\alpha^6\beta_4$ integrin is associated with conditions that favor migration and undifferentiated proliferation of normal keratinocytes and that malignant keratinocytes differ from normal keratinocytes by constitutive phosphorylation of β_4 and by failure to downregulate β_4 transcription in response to calcium in the presence of EGF. [J Natl Cancer Inst Monogr 13:75–86, 1992]

The A9 antigen previously described in our laboratory (*1*) has immunologic and biochemical identity with the newly defined $\alpha^6\beta_4$ integrins (*2–5*). In normal epithelium, the A9/$\alpha^6\beta_4$ antigen is expressed only on the basal surface of basal keratinocytes. Among squamous carcinomas there are three patterns of A9/$\alpha^6\beta_4$ expression. Those squamous carcinomas that exhibit the highest level of A9/$\alpha^6\beta_4$ expression are more likely to recur than squamous cancers that have low expression (*6*). In vitro studies of primary and recurrent cancers from the same patients show that this integrin is one of the few markers that changes with tumor progression (*1*). The factors that result in altered A9/$\alpha^6\beta_4$ expression in the genesis and progression of epithelial carcinomas are unknown. The α^6 and β_4 genes have been cloned and sequenced and provide molecular probes for investigating the genetic basis for altered antigen expression (*7–10*). To elucidate the mechanisms responsible for the patterns of expression that have been observed in epithelial neoplasia, we are using immunofluorescence, Northern blot analysis, and immunoprecipitation of labeled proteins to investigate A9/$\alpha^6\beta_4$ expression in normal and malignant keratinocytes. Because overexpression of the A9/$\alpha^6\beta_4$ integrin may be a factor in the biologic behavior of human squamous carcinomas, a clearer understanding of these mechanisms could lead to the development of novel therapeutic approaches for one of the most common neoplastic diseases in humans.

MATERIALS AND METHODS

Cell Culture

Squamous carcinoma cell lines UM-SCC-8, -14C, and -38 were established in our laboratory from carcinomas of the oral cavity, as described previously (*1*). Tumor cell

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lines were carried in M10 medium, consisting of Dulbecco's modification of Eagle's minimal essential medium supplemented with 2 m*M* L-glutamine, nonessential amino acids (components purchased from Sigma Chemical Co., St. Louis, Mo.), and 10% fetal bovine serum (FBS) (Hyclone, Logan, Utah). Tumor cells were used during midlogarithmic growth unless described otherwise. Subconfluent cultures were harvested with recrystallized porcine trypsin (0.1% wt/vol)(Sigma Chemical Co.) and 0.02% EDTA in Puck's saline A.

Normal keratinocytes were cultivated from skin obtained during elective reduction mammaplasties or from neonatal foreskins, using adaptations of Boyce and Ham's method (*11*). The skin samples were trimmed of fat and subdermal components, minced into small pieces, incubated overnight at 37 °C in collagenase A (2 mg/mL) (Sigma Chemical Co.) and split into dermal and epidermal halves. The keratinocytes were released from the epidermal component by digestion with trypsin-EDTA for 20 minutes at room temperature. After neutralization of the trypsin with M10 or soybean trypsin inhibitor, the keratinocytes were washed twice and resuspended in keratinocyte serum-free medium (SFM) (Life Technologies, Paisley, Scotland) supplemented with 50μg/mL bovine pituitary extract (BPE) and 5 ng/mL epidermal growth factor (EGF) [complete SFM (cSFM)], transferred to plastic flasks, and incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

Compounds known to affect keratinocyte growth and differentiation (calcium, EGF, and all-*trans*-retinoic acid) were assessed for effects on A9/$\alpha^6\beta_4$ expression in normal and malignant keratinocytes. Cells were tested in a basal medium consisting of SFM supplemented with BPE (SFM-B), to which 1.4 m*M* calcium, 5 ng/mL EGF (Life Technologies), and 1 μM all-*trans*-retinoic acid (Sigma Chemical Co.) were added. In one experiment the cells were cultured first in cSFM and then in M10 for 24 hours. In other cases the cells were rinsed and incubated in SFM-B containing test agents for 24 hours. For immunofluorescence experiments, keratinocytes were suspended in cSFM supplemented with 0.5% calf serum (to ensure good attachment) and plated on coverslips in six-well plates. After 12 hours the serum containing medium was removed and the cultures were washed with serum-free MCDB-151 (Sigma Chemical Co.) and fed with cSFM or cSFM containing 1.4 m*M* calcium.

Antibodies

UM-A9 was developed in our laboratory (*1*) and was shown to identify the $\alpha^6\beta_4$ integrin by immunoprecipitation and preclearing experiments (*2*). Monoclonal antibody BQ16 identifies the α^6 protein. Liebert et al. raised BQ16 against human bladder carcinoma cells (submitted for publication). GoH3, a rat anti-α^6 monoclonal antibody (*12*) was generously provided by Dr. A. Sonnenberg. Hybridoma supernatants from standardized batches were used throughout.

Immunofluorescence Assays

Cells grown on glass coverslips, as described previously, were washed with Dulbecco's phosphate-buffered saline (PBS) containing 0.9 m*M* $CaCl_2$ and 0.4 m*M* $MgCl_2$ (PBS-$Ca^{++}Mg^{++}$) (the presence of divalent cations during the assay was essential to prevent cell detachment). The cells were then incubated with normal goat serum, washed, and incubated at 4 °C for 2 hours with UM-A9 hybridoma supernatant (1:10), BQ16 hybridoma supernatant (1:1), or GoH3 rat hybridoma supernatant (1:10), or with isotype-matched control hybridoma supernatants (1B2/B5, a murine IgG; and AIIB2, a rat IgG, kindly provided by Drs. Mark Kaminski and Carolyn Damsky, respectively). After washing again, the cells were incubated with fluorescein or rhodamine conjugated goat antimouse or antirat IgG for 1 hour at 4 °C. After final washings, the cells were fixed with ice-cold acetone for 3–5 minutes, coverslips were mounted, and the cells were examined under the fluorescence microscope. In some experiments the fixed cells were washed in calcium- and magnesium-free Dulbecco's PBS, incubated overnight at 4 °C in rabbit antiactin antibody (Sigma Chemical Co.) (1:1000), washed, and incubated at 23 °C for 1 hour with fluorescein-conjugated goat antirabbit IgG. The coverslips were washed again, mounted on clean glass slides with GVA-mount (glycerol-polyvinyl alcohol and aqueous mounting medium; Zymed Laboratories, San Francisco, Calif.), examined, and photographed using a Nikon immunofluorescence microscope. All sections were photographed at 5-second exposures for fluorescein, and then the same fields were photographed with 10-second exposures using the rhodamine filter.

Cell Labeling

For metabolic labeling, cells were grown to 70%–80% confluence in 35-mm six-well tissue culture plates (Costar), washed in methionine-deficient Dulbecco's modified Eagle's medium (DME), and labeled with 100 μCi/mL [^{35}S]methionine (New England Nuclear) in deficient DME supplemented with 10% dialyzed fetal bovine serum for 4 hours. For experiments in which *N*-glycosylation was blocked, 2 μg/mL tunicamycin or the dimethyl sulfoxide solvent alone was added 3 hours prior to, and during, labeling. Cells were washed twice in PBS with 1 mM cold methionine, lysed in PBS containing 1% Nonidet P-40, 1 m*M* phenylmethylsulfonylfluoride (PMSF), and 1 μg/mL each of leupeptin, aprotinin, soybean trypsin inhibitor, and chymotrypsin inhibitor. The plates were scraped and the lysates were incubated 30 minutes on ice and clarified by centrifugation at 11 000 *g* for 5 minutes.

To assess protein phosphorylation, logarithmically growing cells were labeled with [^{32}P]orthophosphate after 24 hours of growth in M10 or in SFM-B or SFM-B to which 1.4 m*M* $CaCl_2$, 5 ng/mL EGF, or both calcium and EGF were added. The cells were washed three times with phosphate-free physiologic saline (PSS) (*13*) and incubated for 5 minutes in the last wash to reduce the

intracellular free-phosphate pool. New PSS containing 100 μCi/mL of [^{32}P]orthophosphate was added, and the cells were returned to the incubator for 1 hour at 37 °C. The wells were then each washed three times and incubated in the original media (i.e., SFM-B or SFM-B with calcium, EGF, or both) for 30 minutes at 37 °C. The medium was aspirated; the cells were washed three times with cold Dulbecco's PBS with calcium and magnesium; the cells were lysed, scraped, and transferred to a microcentrifuge tube and incubated on ice for 30 minutes to ensure complete solubilization; then the extract was centrifuged at 11 000*g* for 5 minutes.

Immunoprecipitation and SDS-PAGE

Labeled cell extract (containing greater than 2 × 10^6 counts diluted in 100 μL of lysis buffer) was mixed with 50 μL bovine serum albumin (BSA) (10 mg/mL), 150 μL wash buffer containing 1% Nonidet P-40, 50 m*M* Tris-Cl (pH 8), 150 m*M* NaCl, 0.1% sodium deoxycholate, 0.1% SDS, and 1 m*M* PMSF per sample. Samples were precleared twice with 20 μL protein A (Sigma Chemical Co.) or protein G (Pierce Chemical Co.) agarose and incubated overnight at 4 °C with 150 μL of the appropriate antibody. The antigen-antibody complexes were precipitated by incubation with 30 μL of protein A or G agarose for 2 hours at 4 °C, and the precipitates were washed three times with buffer containing 500 m*M* NaCl to reduce nonspecific binding. After a final wash with 150 m*M* NaCl, the pellets were vortexed four times in 0.8 mL wash buffer, pelleted, resuspended in reducing buffer, and separated by SDS-PAGE using a 7% separating gel according to Laemmli's method (*14*). M_r was determined from interpolation based on migration of 200, 116, 95, 66, 45, and 30 kd standards (Sigma Chemical Co.).

Northern Blotting

RNA was isolated from logarithmically growing cells by a modification of the guanidinium thiocyanate procedure (*15*). Cells were washed with ice-cold PBS, placed on ice, and lysed directly in the culture dish with 4 *M* guanidinium thiocyanate containing 25 m*M* sodium citrate, pH 7, 0.5% sarcosyl, and 100 m*M* 2-mercaptoethanol. The mixture was extracted with phenol:chloroform:isoamyl-alcohol and centrifuged at 10 000 *g*. RNA was precipitated from the aqueous phase with isopropanol, washed with 75% ethanol, air dried, redissolved in diethylpyrocarbonate (DEPC)-treated water, and stored at −70 °C. RNA concentration was determined by absorbance at 260 nm and analyzed for β_4 message by Northern blot analysis.

RNA (10 μg/lane) was electrophoresed in 1.2% agarose-formaldehyde gels, stained with ethidium bromide, photographed, and transferred to nylon membranes as described (*16*). Membranes were prehybridized at 42 °C in formamide with denaturated salmon sperm DNA, hybridized at the same temperature with ^{32}P-labeled probes, washed, and autoradiographed. The RNA loading was assessed by visualization of the ethidium bromide-stained gels and by hybridization to a probe for the cyclophilin gene (*17*).

Plasmid DNA containing a 3.8 kb EcoRI fragment for the β_4 chain (cDNA clone P.6.3) (*18*) was kindly provided by M. Hemler. Clone K163 for β_4 (*7*) and clone 1363 for α^6 (*10*) were kindly provided by Dr. A. Sonnenberg. The cyclophilin probe was provided by Dr. Brian Nickoloff with permission from Dr. Matt Harding. Plasmid DNA was extracted from transformed *E. coli* by alkaline lysis (*18*), purified by polyethylene glycol precipitation (*16*), digested with EcoRI (according to the manufacturer's instructions), isolated by electrophoresis in 1% low melt agarose, dissolved in distilled water, and labeled by the random primer method (*19*).

RESULTS

Structural Model and Biochemical Characteristics of the $\alpha^6\beta_4$ Integrin

The α^6 and β_4 genes have been cloned and sequenced (*7–10*). Based on the published sequence data, biochemical data (*2–5,12*), and extracellular matrix binding data (*2, 20,21*), it is possible to construct a model such as the one shown in Fig. 1. Biochemical data correponding to the model are illustrated in Fig. 2. Detergent lysates of the UM-SCC-38 cell line, a high expressor of the A9 antigen, labeled with [^{35}S]methionine (lanes 1, 2) or [^{32}P]orthophosphate (lanes 3, 4), were immunoprecipitated by UM-A9 antibody and protein A sepharose. As shown in lane 1, and previously reported in (*2*), UM-A9 precipitates the 205, 175, 140, and 125 kd proteins typical of the $\alpha^6\beta_4$ integrin heterodimer (*3–5*). Incubation of the tumor cells with tunicamycin blocks glycosylation of the α^6 and β_4 chains, as reported previously (*2–5*). When extracts of [^{32}P]orthophosphate-labeled UM-SCC-38 tumor cells are precipitated with the UM-A9 antibody, only the 205 kd species is labeled. This is consistent with the previous reports by Kennel et al. (*3*) and Sacchi et al. (*22*). UM-A9 does not identify the $\alpha^6\beta_4$ complex in Western blots, but both the GoH3 antibody to α^6 developed by Sonnenberg et al (*12*) and the anti-α^6 specific BQ16 antibody raised to bladder carcinoma cells by Liebert et al. (submitted for publication) detect the 125kd α^6 protein on blots of UM-SCC-38 cell extracts (lanes 5, 6).

Regulation of A9/$\alpha^6\beta_4$ Integrin Expression by Culture Conditions

As we reported previously, strong nonpolar expression of the A9/$\alpha^6\beta_4$ antigen is observed in highly malignant keratinocytes in vivo and in vitro (*1,6*). We wished to determine the basis for the association of abnormal expression of this integrin with aggressive biologic behavior of squamous cancers by examining factors that regulate expression in normal keratinocytes. A key in vitro difference between normal and malignant keratinocytes is the ability of malignant or transformed cells to continuously proliferate in calcium-containing growth medium such as M10. In contrast, normal keratinocytes require low-

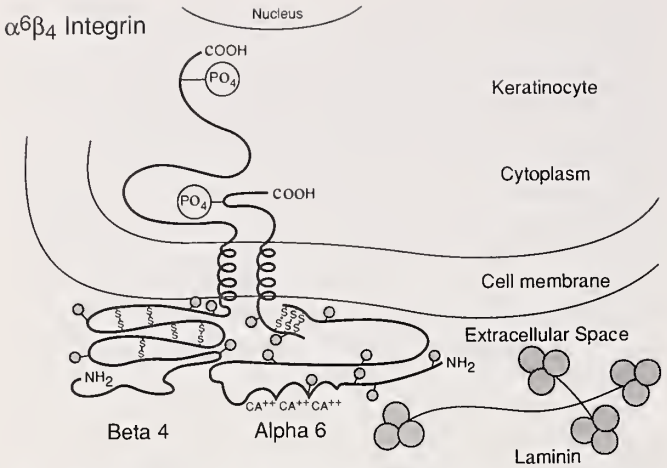

Fig. 1. Hypothetical model of the $\alpha^6\beta_4$ integrin. This model, modified from Kennel et al (*30*), is based on the data of Hogervorst et al (*7*), Suzuki and Naitoh (*8*), Tamura et al (*9*) and Hogervorst et al (*10*). Where there were differences between different authors, this model follows the data of Hogervorst et al (*7,10*), who cloned the α^6 and β_4 genes from a normal keratinocyte library. The figure shows the β_4 chain on the left and the α^6 chain on the right. The β_4 chain consists of ~1778 amino acids, of which 683 are in the extracellular space, 23 make up the transmembrane domain, and 1072 make up the intracellular portion. The extracellular domain contains a cysteine-rich domain from which many possible intrachain disulfide bridges are possible. There are five possible *N*-linked glycosylation sites (amino acid sequence asn-X-ser/thr) in this domain that are marked by shaded circles. The β_4 chain contains a phosphorylation site located near the c-terminus (*3,30*). The α^6 chain consists of 1050 amino acids: 991 in the extracellular domain, 23 in the transmembrane domain, and 36 in the intracellular domain (*9,10*). The α^6 chain is synthesized as a single polypeptide and then cleaved to a light chain containing the c-terminal intracellular domain and the transmembrane domain, as well as a short extracellular domain that is covalently linked by disulfide bridges to the larger extracellular domain. The α^6 sequence also contains three (possibly four) consensus calcium-binding domains and nine sites of potential *N*-linked glycosylation—two on the light chain and the remainder on the heavy chain. A laminin-binding domain is indicated near the amino terminus based on data from Sonnenberg et al (*20*), Lotz et al (*21*), and Van Waes et al (*2*). We aligned the calcium-binding domains as possible anchoring sites based on our observations that EDTA is required for efficient detachment of A9/$\alpha^6\beta_4$ positive squamous carcinomas.

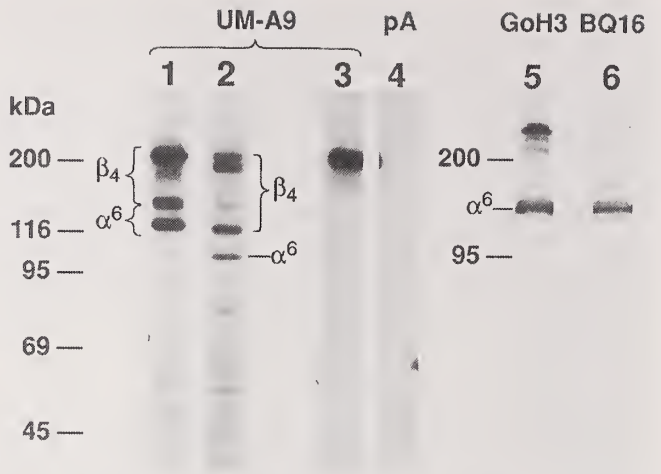

Fig. 2. Biochemical characteristics of the $\alpha^6\beta_4$ integrin in squamous carcinoma cells. Autoradiogram (lanes 1, 2) showing A9/$\alpha^6\beta_4$ proteins from UM-SCC-38 cells metabolically labeled with [^{35}S]methionine and isolated by immunoprecipitation with UM-A9 antibody and protein A sepharose separated by SDS-PAGE under reducing conditions. Lane 2 contains immunoprecipitated extracts from identically labeled UM-SCC-38 cells cultured in the presence of tunicamycin, an inhibitor of *N*-glycosylation. Lanes 3 and 4 contain extracts of UM-SCC-38 cells labeled with [^{32}P]orthophosphate and precipitated with UM-A9 (lane 3) and protein A sepharose or with pA sepharose alone (lane 4). Lanes 5 and 6 show Western blots of UM-SCC-38 cell extracts separated by SDS-PAGE under reducing conditions and immunostained with the anti-α^6 monoclonal antibodies GoH3 (*12*) and BQ16 (Liebert et al, submitted for publication).

calcium medium supplemented with growth factors for continued in vitro clonal proliferation (*11*). We previously reported (*23*) that normal keratinocytes growing under these conditions exhibit high $\alpha^6\beta_4$ expression at the transcriptional level, with message levels that exceed those found in malignant cells cultured in conventional high-calcium, serum-containing medium. Thus, under conditions in which normal keratinocytes behave like tumor cells, that is, continuous replication and migration, they also express high levels of an integrin strongly expressed by tumor cells. To understand how normal and malignant cells differ with respect to $\alpha^6\beta_4$ expression and regulation, we first examined how normal cells respond to the conditions under which tumor cells are usually cultivated.

Normal keratinocytes in logarithmic growth were grown on coverslips for 3 days in cSFM. They were then fed with either M10 or fresh cSFM for 24 hours and examined for α^6 and β_4 antigen expression by immunofluorescence using BQ16 and UM-A9 antibodies. The results are shown in Fig. 3. The cells in the low-calcium growth factor-supplemented medium (cSFM) show numerous filopodia with strong staining by UM-A9 (Fig. 3,A) and BQ16 (Fig. 3,C) at all cell borders. After 24 hours in the M10 medium containing serum and high calcium (Fig. 3,B,D), the cells have formed intercellular attachments and exhibit fewer and shorter filopodia, with staining visible by both antibodies only at the areas not covered by cells. Areas of stained material are apparent on the glass, as though in retracting into islands, the cells have left filopodia "footprints." Thus, within 24 hours, serum and calcium containing medium induced changes in keratinocyte morphology and antigen expression that are compatible with antigen polarization and induction of differentiation. To dissect the numerous differences between cSFM and M10 and to compare the responses of normal and malignant keratinocytes to calcium alone, we performed the next series of experiments in cSFM.

Calcium Rapidly Induces Polar A9/$\alpha^6\beta_4$ Expression in Normal but not Malignant Keratinocytes

Normal keratinocytes (Fig. 4,A,B) and two squamous carcinomas—UM-SCC-8 (Fig. 4,C,D) and UM-SCC-38 (Fig. 4,E,F)—from logarithmically growing cultures were grown on glass slides as described and then fed either with

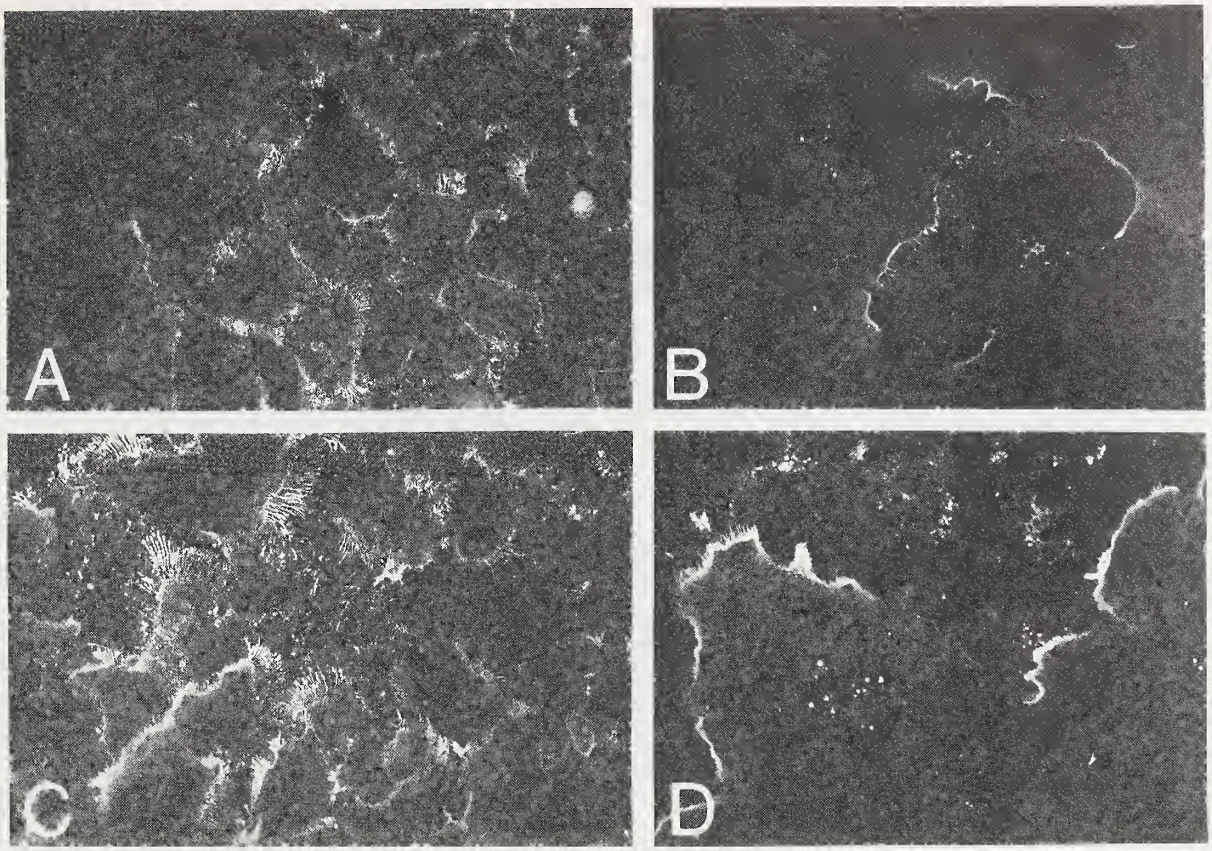

Fig. 3. Changes in the $\alpha^6\beta_4$ expression in normal keratinocytes after 24 hours in M10. Normal human keratinocytes cultured for 3 days on coverslips in cSFM were fed with fresh cSFM (A,C) or M10 medium (B,D) and stained with monoclonal antibodies UM-A9 (anti-β_4) (A,B) or BQ16 (anti-α^6) (C,D) using a rhodamine conjugated goat antimouse IgG antibody. Panel A, 100× objective, panels B-D, 50× objective.

cSFM or with cSFM containing 1.4 mm calcium chloride. In all three cultures, those fed with calcium (Fig. 4,B,D,E) exhibited increased spreading and cell-cell contact that was more pronounced in the normal keratinocytes (Fig. 4,B) and in UM-SCC-8 (Fig. 4,D). In identical cultures shown in Figs. 5 and 6, A9/$\alpha^6\beta_4$ antigen expression was significantly reduced, as shown by the intensity of staining by UM-A9 in the normal keratinocytes after 24 hours of exposure to calcium (compare Fig. 5,B to Fig. 6,B). In the tumor cells the reduction was slight (compare Fig. 5,D and 5,F to Fig. 6,D and 6,F).

Calcium and EGF Stimulate β_4 Messenger RNA Expression in Tumor Cells

In previous experiments we had shown that normal and malignant keratinocytes have equivalent levels of α^6 and β_4 messenger RNA in cSFM, but in M10, malignant keratinocytes had relatively less message. To determine if the difference in message expression was due to the calcium level, we examined two tumor cell lines, UM-SCC-38 and UM-SCC-14C, that exhibit high A9 antigen expression in conventional medium. The tumor cells were given 24 hours of exposure to SFM-B to which calcium alone, EGF alone (cSFM), or EGF and calcium were added; then the cells were lysed and β_4 messenger RNA levels were compared. As shown in Fig. 7,A and reported previously (*23*), β_4 message in EGF was comparable to that in the control cultures (less than 2% difference by densitometry). When calcium was added to the basal medium, β_4 message decreased moderately in UM-SCC-38 (73% of control) and increased moderately in UM-SCC-14C (140% of control). But when calcium and EGF were present together, β_4 message increased to 172% of control in UM-SCC-38 and 300% of the SFM-B control in UM-SCC-14C. These results are consistent with the failure of these tumor lines to exhibit polar expression of the A9/$\alpha^6\beta_4$ complex or to undergo differentiation in response to calcium in EGF-containing medium. It will be important to determine how β_4 message in normal keratinocytes responds to these conditions. Those experiments are now underway.

Retinoic Acid Stimulates β_4 Expression in Serum-Free Medium in the Absence of EGF

Retinoic acid is a compound with striking effects on epithelial cells that include both induction of differentiation (*24,25*) and stimulation of the proliferative response of keratinocytes to growth factors (*26*). It is also one of the first agents shown to have the potential for preventing new head and neck cancers (*27*).

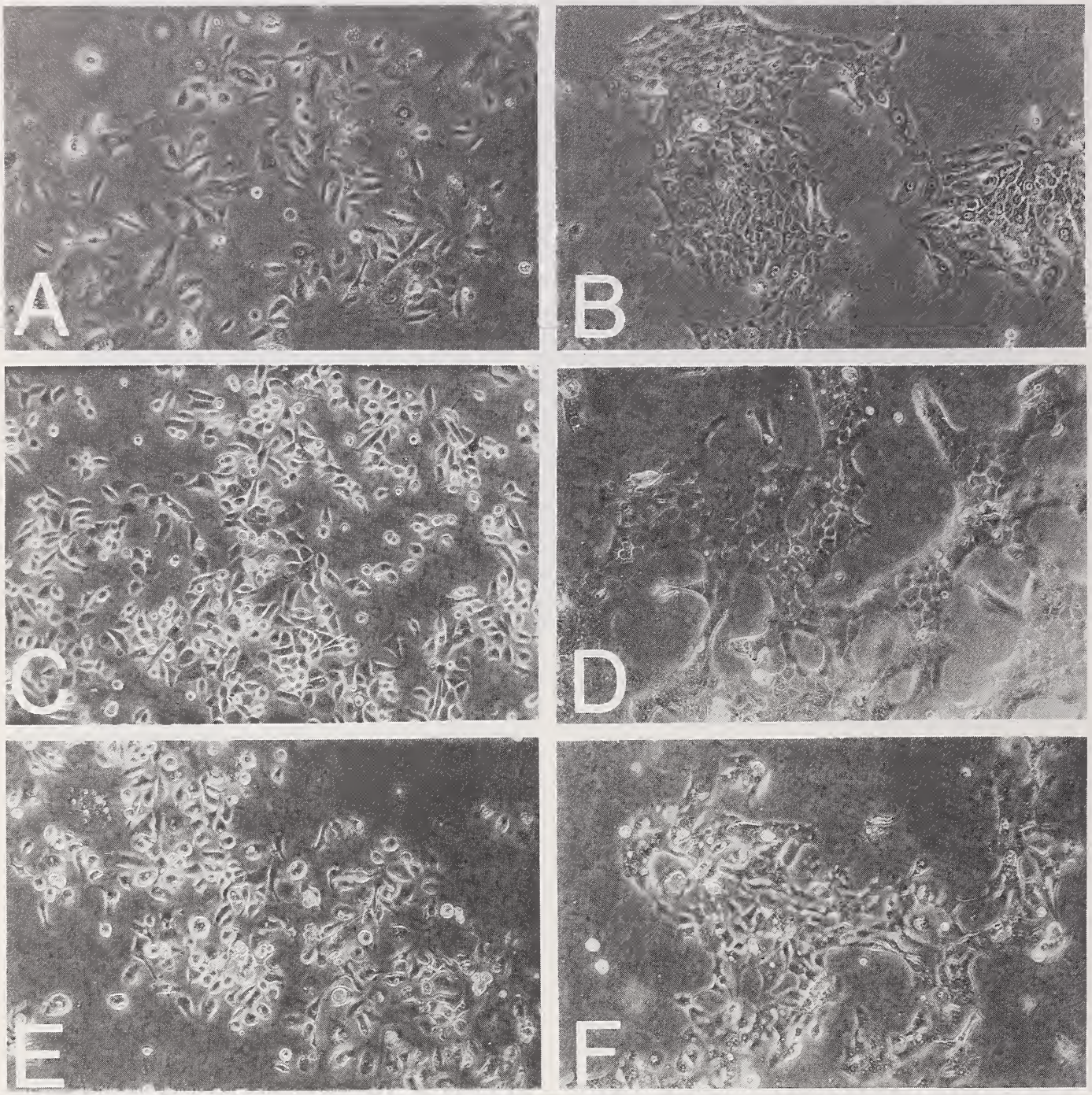

Fig. 4. Inverted phase contrast photomicrographs of normal and malignant keratinocytes after 24 hours in cSFM (A,C,E) and cSFM containing 1.4 m*M* calcium (B,D,F). Normal keratinocytes (A,B); UM-SCC-8 (C,D); UM-SCC-38 (E,F). All photos 10× phase contrast objective.

In the same experiment as that discussed previously, we tested retinoic acid for effects on β_4 expression. As shown in Fig. 7,B, 24 hours of incubation with retinoic acid strongly stimulated transcription of β_4 message in both UM-SCC-38 and -14C. The concurrent presence of calcium partially blocked the increase in both tumor lines. These findings suggest that experiments to determine the effects of retinoids on cell growth and migration in the presence or absence of calcium are indicated.

Constitutive β_4 Phosphorylation Distinguishes UM-SCC-38 Cells from Normal Keratinocytes

Normal keratinocytes and UM-SCC-38 cells were grown for 24 hours under conditions known to alter expression

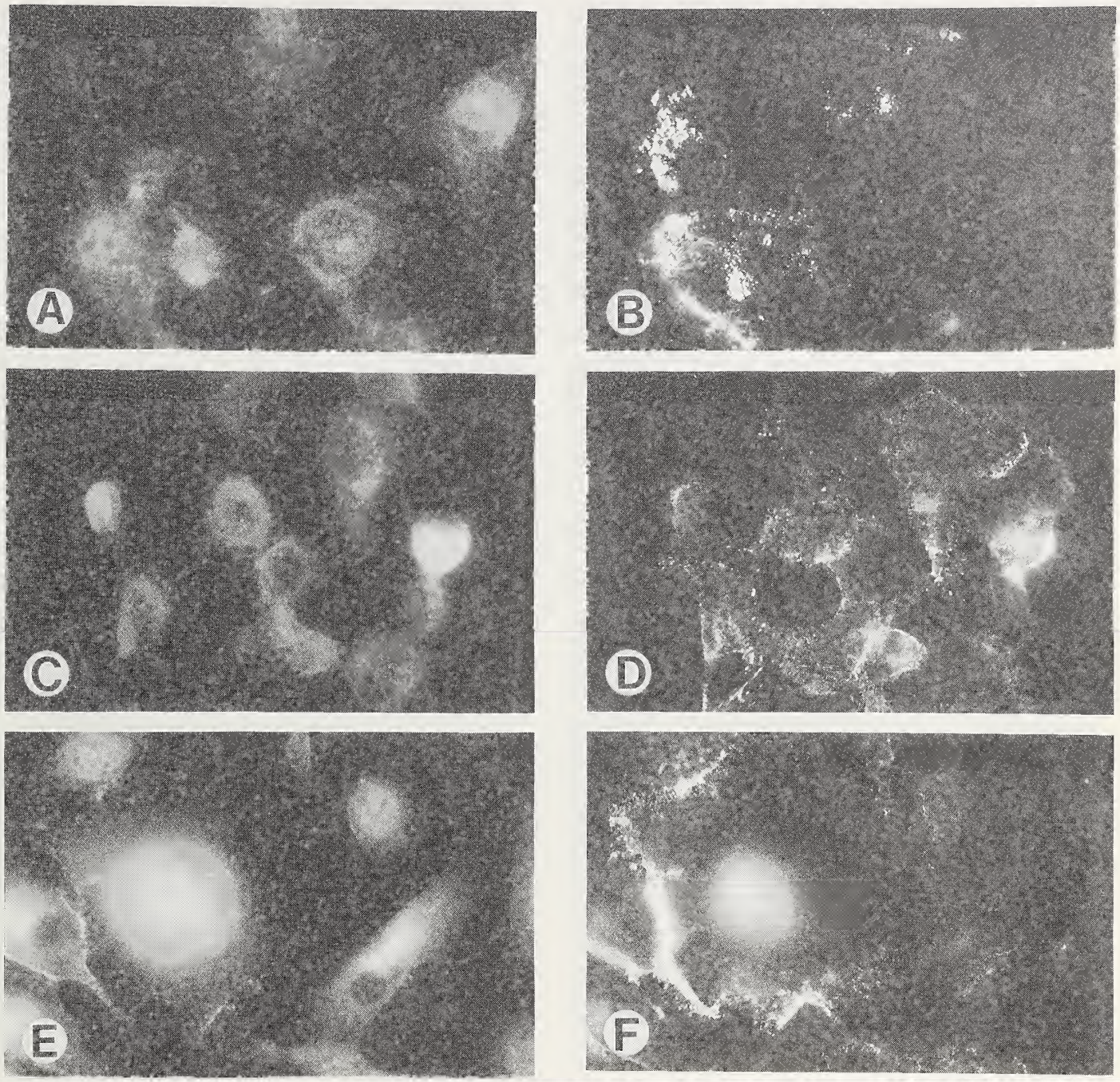

Fig. 5. Immunofluorescence photomicrographs of normal and malignant keratinocytes grown in cSFM. Normal keratinocytes (A,B), UM-SCC-8 (C,D), and UM-SCC-38 (E,F) were grown on coverslips for 12 hours in cSFM with 0.5% calf serum; were rinsed and fed with cSFM for 24 hours; and were fed again for 24 hours with cSFM. They were then stained for A9/$\alpha^6\beta_4$ antigen expression using antibody UM-A9 and rhodamine conjugated goat antimouse IgG. Cells were then fixed and stained with rabbit antiactin and fluorescein conjugated goat antirabbit IgG. Each coverslip was photographed using the fluorescein filter (A,C,E) to identify the location of the cells in each field with the actin staining. The filter was switched to the rhodamine excitation wavelength, and the same field was photographed again to show the β_4 staining. All photos were taken with the 100× objective.

of A9/$\alpha^6\beta_4$ in normal keratinocytes and then were tested for β_4 phosphorylation, as described previously. As shown in Fig. 8, β_4 phosphorylation in normal keratinocytes was relatively low in SFM-B but increased 30% in EGF alone and twofold in response to calcium or calcium plus EGF. In contrast, phosphorylation of β_4 in UM-SCC-38 was high in the basal medium and fluctuated by less than 25% in any of the conditions tested.

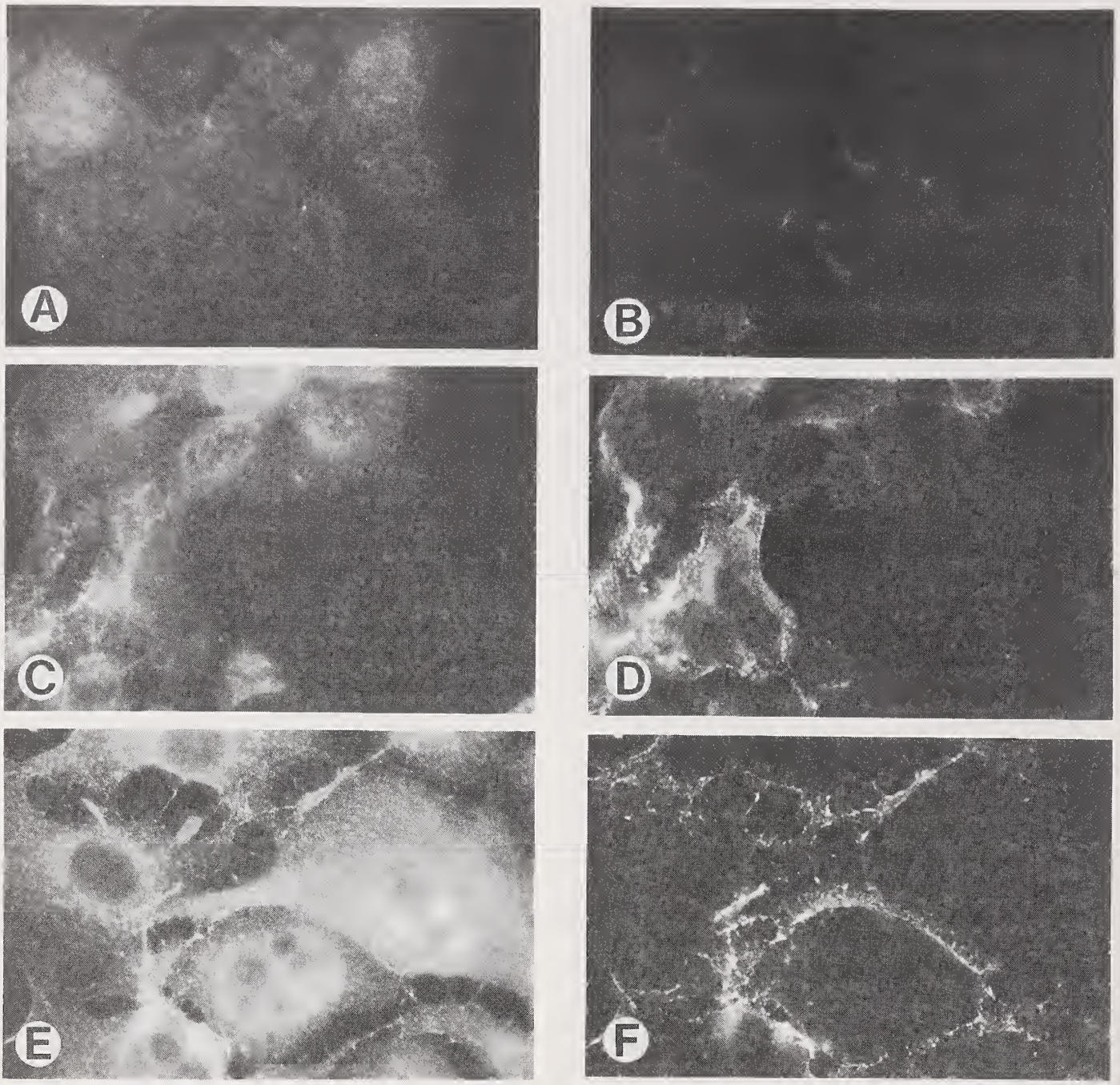

Fig. 6. Immunofluorescence photomicrographs of normal and malignant keratinocytes grown in cSFM containing 1.4 m*M* calcium. Normal keratinocytes (A,B), UM-SCC-8 (C,D), and UM-SCC-38 (E,F) were cultured exactly as described in Fig. 5 except that the cells were grown for the last 24 hours before staining in cSFM containing calcium. Cells were stained and photographed as described in Fig. 5.

DISCUSSION

The A9 antigen (*1*) is a marker of highly malignant behavior in squamous cell carcinoma of the head and neck (*6*). We have recently shown the A9 antigen to be an $\alpha^6\beta_4$ integrin with laminin-binding activity (*2*). In this report we reviewed the biochemical characteristics of the A9/$\alpha^6\beta_4$ integrin and presented a model of the heterodimer structure that can be used to predict structure function relationships. Although the α^6 protein has been shown in several cell types to have laminin-binding activity when associated with the β_1 polypeptide (*28*), there is some controversy over its function as a laminin receptor in the $\alpha^6\beta_4$ complex. Sonnenberg et al (*20*) found no laminin-binding activity of α^6 in an $\alpha^6\beta_4$ positive cell line that failed to express $\alpha^6\beta_1$. In contrast, Lotz et al (*21*) and our group (*2*)

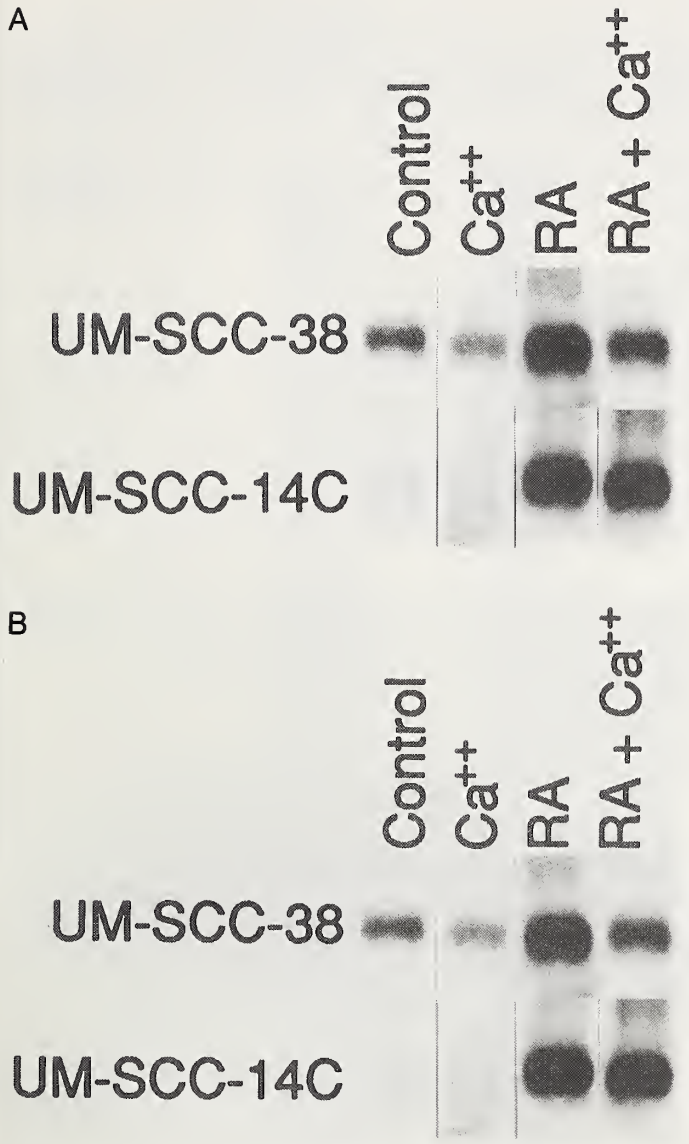

Fig. 7. Northern blot analysis showing affects of epidermal growth factor, calcium, and all-*trans*-retinoic acid on β_4 mRNA in high A9/$\alpha^6\beta_4$ expressor SCC lines. A) RNA extracted from logarithmically growing UM-SCC-38 and UM-SCC-14C cells cultured for 24 hours in SFM-B medium (control), in SFM-B medium to which 1.4 m*M* calcium was added (Ca^{++}), in cSFM (SFM-B plus 5 ng/mL EGF) (EFG), and in cSFM to which 1.4 m*M* calcium was added (EGF + Ca^{++}) was probed for β_4 message as described in Materials and Methods. B) Northern blot of β_4 mRNA from UM-SCC-38 and UM-SCC-14c cells grown for 24 hours in SFM-B (control), in SFM-B plus 1.4 m*M* calcium (Ca^{++}), in SFM-B plus 1 μM retinoic acid (RA), or in SFM-B with both calcium and retinoic acid (RA + Ca^{++}).

have found that anti-α^6 antibody can block laminin binding in tumor cell lines that express $\alpha^6\beta_4$ but not $\alpha^6\beta_1$.

On this basis we conclude that at least part of the function of this integrin is to bind to the extracellular matrix through an interaction with laminin. In the model, we placed the calcium-binding sites in an orientation that suggests an anchoring function in the presence of calcium. This is consistent with the induction of polar A9/$\alpha^6\beta_4$ expression and decreased mobility of normal keratinocytes in the presence of high calcium concentrations (Figs. 4-6). The function of the β_4 chain is not known, but its ability to be phosphorylated (*3,22*) suggests that it may have a signal transducing capacity. Shaw et al (*29*) have reported that the α^6 light chain is also phosphorylated, but we have not yet studied this possibility.

Because overexpression of A9/$\alpha^6\beta_4$ is frequently observed in squamous carcinomas and is associated with aggressive clinical behavior, we want to determine the basis for overexpression. One simple hypothesis is that gene amplification of either the α- or the β-chain genes occurs during tumor progression. Thus far we have only preliminary results with Southern blots of tumor cell DNA analyzed with the β_4 probe, and no examples of gene amplification have been found.

A second hypothesis is that overexpression of the antigen is the result of constitutive expression of α^6 and β_4 message. Our first experiments compared messenger RNA from tumor cells and normal keratinocytes growing in M10 and cSFM media, respectively. The results from these experiments showed that culture conditions can regulate expression in both normal and tumor cells. When normal keratinocytes are grown under conditions that foster rapid clonal proliferation, that is, EGF and low calcium, then $\alpha^6\beta_4$ expression at the transcriptional level exceeds that of even some highly malignant cells cultured in conventional calcium and serum-containing medium (*23*). We also found that message expression in tumor cells increased in the serum-free, low-calcium keratinocyte medium. Removing EGF from the medium had little effect on β_4 message, and this led us to begin to investigate the effects of calcium, since calcium is one of the key factors that affects normal keratinocyte differentiation in vitro.

Although calcium induced down-regulation of expression of the A9/$\alpha^6\beta_4$ complex on the cell surface in normal cells, the effect on malignant cells was much less pronounced, even when we tested UM-SCC-8, a cell line that at confluence exhibits polar expression of the A9 antigen (*1*). Consistent with this finding, tumor cells cultured in high calcium exhibited only minimal reduction in β_4 message expression. An unexpected finding was that high calcium in the presence of EGF led to increased β_4 expression. Nevertheless, this is consistent with the high level of A9 antigen expression usually observed in calcium-containing medium supplemented with serum that presumably contains EGF. These findings indicate the need for similar analysis of normal keratinocyte message.

Of particular interest was the finding that retinoic acid strongly stimulates transcription of β_4. Retinoic acid is known to stimulate transcription of laminin (*25*) in the F9 murine teratocarcinoma, but the effect occurs relatively late (72 hours) after retinoic acid exposure. It is therefore of interest that β_4 is a relatively early response gene for retinoic acid and one that may serve as an intermediate signal for laminin expression. Generally, we associate high $\alpha^6\beta_4$ expression with unrestrained proliferation and migration in either normal or malignant cells. Retinoic acid has

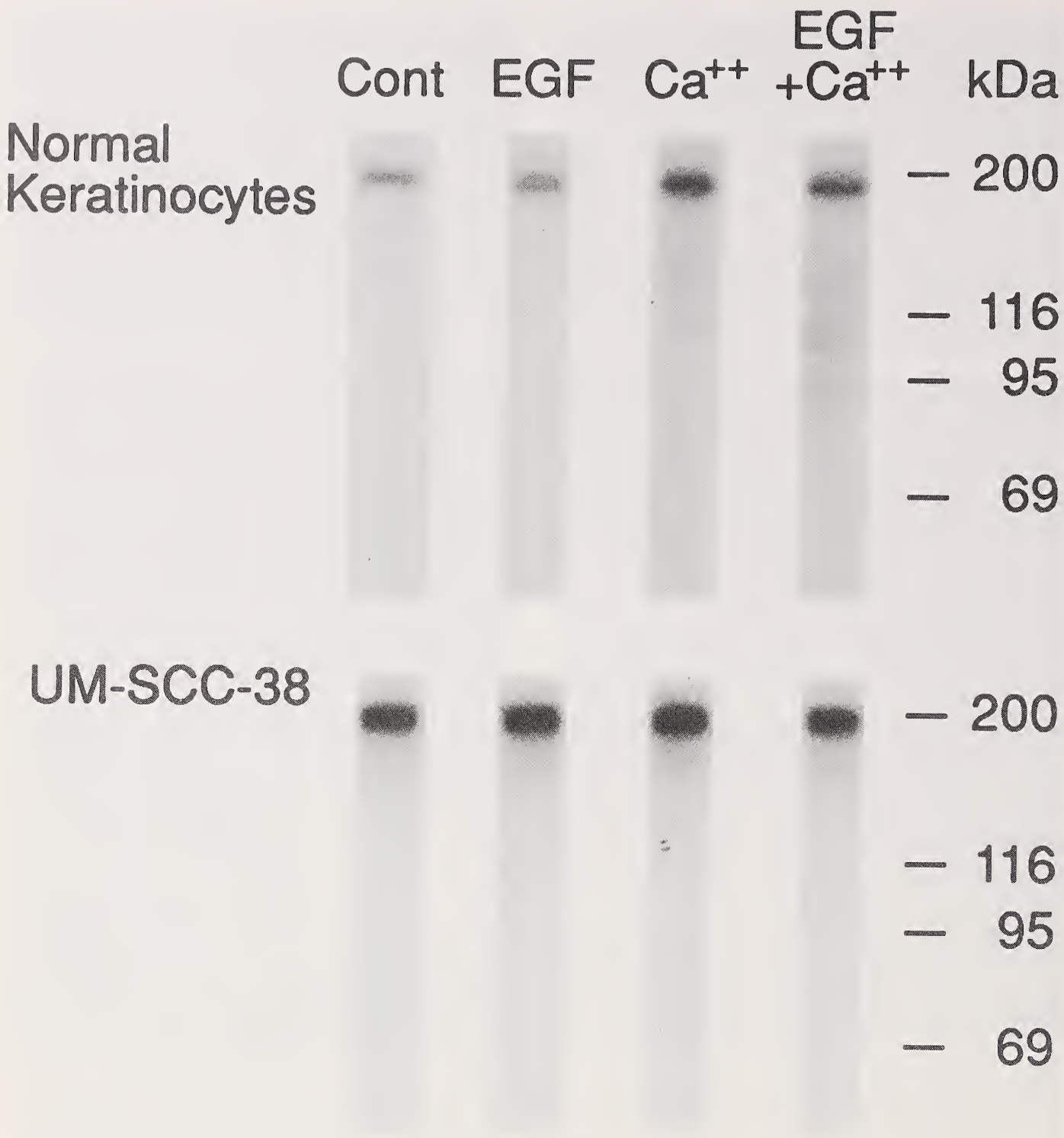

Fig. 8. Phosphorylation of β_4 in normal and malignant keratinocytes. Autoradiograms of [^{32}P]-labeled extracts precipitated from cells grown for 24 hours in SFM-B (control), in cSFM (EGF), in SFM-B plus 1.4 m*M* calcium (Ca^{++}), or in cSFM plus 1.4 m*M* calcium (EGF + Ca^{++}).

a stimulatory effect on normal keratinocyte proliferation in the presence of EGF (*26*) in low-calcium medium. It is reasonable that the increased β_4 expression may be part of the cascade in the proliferative response. That calcium down-regulates the stimulatory effect of retinoic acid on β_4 expression is consistent with the possibility that together the two stimuli lead to epithelial differentiation. Clearly, this is an area that deserves further investigation.

One of our most striking findings is the observation that 200 kd β_4 protein may be constitutively phosphorylated in squamous carcinomas. If phosphorylation is a signaling mechanism associated with keratinocyte differentiation, then unlinking the receptor function of the $\alpha^6\beta_4$ integrin and its phosphorylation state might block differentiation and lead to unrestrained proliferation. This is an area for further investigation.

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Keratinocyte Differentiation Markers: Involucrin, Transglutaminase, and Toxicity[1]

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ABSTRACT—Studies of three keratinocyte differentiation markers are described. First, the involucrins of several mammals are identified, facilitating use of this marker in animal models of human disease. The rapid evolution of involucrin has prevented its routine immunochemical identification beyond the primates, but its unusual solubility and its labeling by transglutaminase have permitted detection in rats, cats, and sheep. Second, the re-expression of keratinocyte transglutaminase in carcinoma cells lacking the enzyme is demonstrated. Lack of this enzyme expression has been observed previously in squamous cell carcinomas. The present finding suggests genomic hypermethylation could contribute to this phenomenon and offers an approach to analyzing transcriptional features of the enzyme regulation. Third, the sensitivity of keratinocytes to growth suppression by aflatoxin B_1 is reported. The observed toxicity appears to be mediated by aryl hydrocarbon hydroxylase, a metabolic enzyme inducible in keratinocytes by environmental agents. Such expression may be relevant to carcinogenesis in tissues subject to squamous metaplasia as well as in other exposed cell types stimulated to express this biotransformation enzyme. [J Natl Cancer Inst Mongr 13:87-91, 1992]

Analysis of the perturbed differentiation in cells at various stages of neoplasia may reveal at least three types of useful information. First, abnormal properties of the cells may correlate well with certain stages of the disease or ultimate prognosis and thus have relevance to clinical diagnosis. A given differentiation marker may be of limited value alone but may be informative as part of a battery of indicators. Second, a marker may serve in a targeting process for therapeutic agents. For this purpose, marker antigens appearing on cell surfaces or enzymes activating chemotherapeutic agents are likely to be helpful. Because complete distinction between target and nontarget cells is unlikely for a single marker, exploiting combinations of such markers may be important to assure specificity. Third, if a marker protein contributes substantially to the neoplastic properties a cell displays, understanding the basis for its disturbed regulation may have therapeutic value. Even if it does not contribute (a more common situation), perturbation of its expression is a manifestation of disturbance in regulatory processes. Thus, describing the defective regulation that results in aberrant expression may be critical to understanding the pathway from initial cellular insult ultimately to malignancy. To this end, improvements in understanding cellular information transduction processes are anticipated to be of assistance and to be stimulated by such endeavors.

Among the increasing number of markers useful in keratinocyte studies, two specific for this cell type are of considerable interest. A membrane-bound transglutaminase (TG_K) and a major substrate (involucrin) are synthesized in abundant amounts, primarily in the upper spinous cells of stratified squamous epithelia (*1*). Each has been molecularly cloned (*2,3*), facilitating regulatory studies. These markers are also expressed in cells undergoing squamous metaplasia and in squamous carcinomas from these and other tissues. The keratinocyte reprogramming that occurs during squamous metaplasia presumably endows the cells with other biochemical attributes, including biotransformation capability by inducible aryl hydrocarbon hydroxylase (CYPIA1). Thus, examining the toxic response of keratinocytes to agents that are activated by this metabolic enzyme may have relevance for target sites such as the lung in regions where this activity ordinarily is inducible or where keratinocyte properties arise.

MATERIALS AND METHODS

Cell Culture and Treatments

Unless specified otherwise, keratinocytes were cultivated with 3T3 feeder layer support in a 3:1 mixture of Dulbecco's modified Eagle's medium and F12 media supplemented with 1 μM hydrocortisone and 5% fetal bovine serum (*4*). Keratinocytes grown from normal tissues were started as explants with feeder layer support and maintained in the medium above, supplemented with epidermal

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growth factor and cholera toxin (each 10 ng/mL). One day after inoculation, SCC-4 cells were exposed to 5-azacytidine (1–5 μM) for a day, after which the medium was changed and the cells permitted to grow for an additional 2–3 weeks. Cultures were then stained for TG_K using B.C1 monoclonal antibody by immunogold-silver methodology (*5*). For toxicity measurements, SCC-9 cells without a feeder layer were inoculated at 2×10^5 per 6-cm dish in medium containing 2% serum. Starting with a medium change after 2 days, the cells were maintained in 1% serum and the agent of choice or solvent alone (0.1% dimethyl sulfoxide final concentration). After two subsequent medium changes at 4-day intervals (day 14), the cells were trypsinized and counted.

Involucrin Identification

Stored frozen until use, explant and first-passage cultures were homogenized in 10% trichloroacetic acid. Insoluble material was rinsed in 5% trichloroacetic acid, resuspended in water, adjusted to pH 8 with redistilled *N*-ethylmorpholine, and adjusted to 67% ethanol. After clarification, the material remaining soluble (enriched for involucrin) was sampled directly for electrophoresis in 7.5% acrylamide gels (*6*) and in some cases transferred to nitrocellulose (*7*) for immunochemical staining. In other experiments, the enriched involucrin fraction was extracted with amyl acetate, and the aqueous (lower) phase thus depleted of ethanol was lyophilized. The lyophilized material was resuspended in Tris-Cl buffer (pH 8), clarified and reacted with human TG_K (solubilized from culture particulates with nonionic detergent) in the presence of 5 mM calcium ion and ^{14}C-glycine ethyl ester (*8*). The reaction mixture was then electrophoresed as above and the gels stained with Coomassie blue, dried, and autoradiographed. Antihuman involucrin antiserum was that previously described (*9*), and antidog involucrin antiserum was kindly supplied by Dr. Marcia Simon (*8*).

RESULTS

Nonhuman Involucrins

Fig. 1 illustrates the involucrins detected in keratinocytes from the human and several other mammals. The procedure employed takes advantage of the striking stability of involucrin to denaturing conditions. In general, the cleanest preparations were obtained from keratinocytes cultured from stratified squamous epithelia. Thus, culture extracts were treated with 10% trichloroacetic acid and neutralized, and then the resolubilized material was treated with two volumes of ethanol, thereby denaturing the large majority of cell protein. Alternately, the extracts were boiled for 3 min (*10*) and clarified by low-speed centrifugation after addition of two volumes of ethanol. The latter procedure was especially advantageous for tissues (e.g., Fig. 1*a*), since boiling improved subsequent homogenization and resulted in lower residual background material.

Samples from sheep gave two bands of involucrin (Fig. 1*a,b*) with mobility similar to that from chimpanzee (Fig. 1*c*) and human. Some primate species routinely give two bands of involucrin (*11*), which is attributable to allelic polymorphism, a phenomenon recently detected in the human population (*12*). Chimpanzee involucrin is highly cross-reactive with antihuman involucrin antiserum (*11*). Although producing bands of approximately 5% the intensity of the same amount of human involucrin, the sheep protein was clearly cross-reactive with such antisera

Fig. 1. Detection of involucrin by solubility properties, by immunochemical cross-reactivity, and as a transglutaminase substrate. Separate experiments are grouped. Sample *a* was obtained from esophageal mucosa, and the others were obtained from epithelial cells cultured from esophagus (sheep, cat), epidermis (human, rat), or vagina (chimpanzee). Chimpanzee (*c*) and sheep (*a,b,d*) involucrins were stained with Coomassie blue (*a–c*) or anti-involucrin antiserum (*d*). Human (*f,i*), cat (*g,j*), and rat (*h,k*) involucrins were stained with Coomassie blue (*f–h*) or labeled with ^{14}C-glycine ethyl ester and autoradiographed (*i–k*). The marker protein phosphorylase a (*e*) was electrophoresed in parallel and detected with Coomassie blue. Placement of stained gels (as in *f–h*) on autoradiographs (as in *i–k*) in given experiments showed a coincidence of the bands detected by Coomassie blue stain and radioactive labeling.

in immunoblotting experiments (Fig. 1*d*). Rat and cat involucrins exhibited considerably faster electrophoretic migration than did the human protein or the marker protein, phosphorylase a (Fig. 1*e–h*). Neither was detectable by immunoblotting with anithuman antisera (although in preliminary experiments the rat protein was detected using antidog involucrin antiserum). However, both were labeled by transglutaminase with radioactive glycine ethyl ester, as was the human protein (Fig. 1*i–k*). The sheep protein could also be labeled in this way (M. Simon, personal communication). Corroborating evidence of this kind can be important, since in some samples substantial amounts of protein of similar (likely collagen) or lower molecular weight are observed. In parallel experiments, as previously observed (*11*), late-passage rat keratinocytes, which undergo spontaneous immortalization (*13*), did not express detectable involucrin.

5-Azacytidine-Induced TG_K

TG_K ordinarily is abundantly expressed in differentiating keratinocytes cultured from normal human epidermis or squamous carcinomas (*14*) or serially cultivated epithelial cells derived from a variety of rat tissues (*5*). However, the SCC-4 line, derived from a lingual squamous cell carcinoma (*15*), exhibits little or none of this membrane-bound enzymatic activity (*14*). As shown in Fig. 2, the cells also lacked detectable TG_K immunoreactivity. However, after treatment with 5-azacytidine, immunoreactivity was readily observed in a considerable fraction of the cells (Fig. 2). Whether expression of other markers of keratinocyte differentiation is altered in such cells has not yet been tested in these cultures. However, 5-azacytidine has been reported to induce re-expression of involucrin in SV40-transformed human epidermal cells (*16*). The effect of such treatment on differentiation of adenocarcinomas or small-cell carcinomas of the lung would also be of interest.

Aflatoxin Toxicity

When treated with polycyclic aromatic hydrocarbons such as 3-methylcholanthrene, SCC-9 squamous carcinoma cells are greatly inhibited in growth (*17*). Compatible with its ability to induce aryl hydrocarbon hydroxylase and to be activated to toxic metabolites, benzo(a)pyrene also is a potent inhibitor of the growth of these cells (Fig. 3). In comparison, aflatoxin B_1 is as powerful at high concentration but appears less potent. The simplest explanation for this observation is that the mycotoxin is only a weak inducer of the responsible metabolic activity but is metabolized to a highly toxic product(s). More recent experiments support this interpretation. Indeed, in keratinocytes derived from normal epidermis, aflatoxin B_1 shows a similar concentration dependence to that illustrated. However, 5 n*M* tetrachlorodibenzo-p-dioxin (TCDD), a highly effective inducer of aryl hydrocarbon hydroxylase, increases its potency by at least an order of magnitude (*18*). TCDD itself does not affect the growth rate under the conditions employed, nor does it affect the lack of toxicity of aflatoxin B_1 dihydrodiol or aflatoxin B_2.

DISCUSSION

Initial attempts to detect involucrins in rodent keratinocytes by immunochemical cross-reactivity with antihuman involucrin antiserum gave negative results. This proved advantageous for certain purposes, such as demonstrating the persistence of human skin grafts on the mouse (*19*). Subsequent study showed that even within the primate order, this protein has undergone rapid evolution,

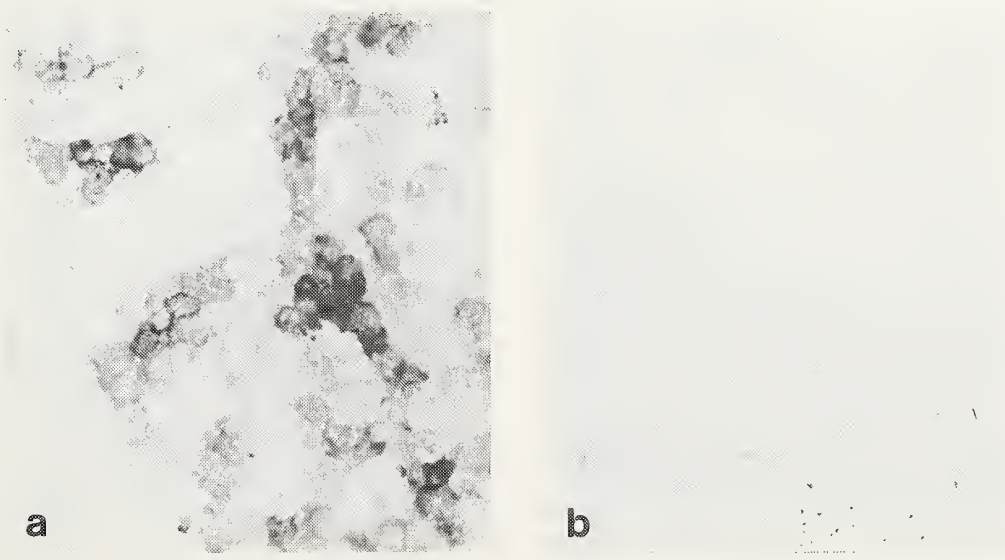

Fig. 2. Induction of TG_K in squamous carcinoma cells (SCC-4) by 5-azacytidine. The treated cells (a) have readily detectable TG_K, whereas none is seen in parallel untreated controls (b).

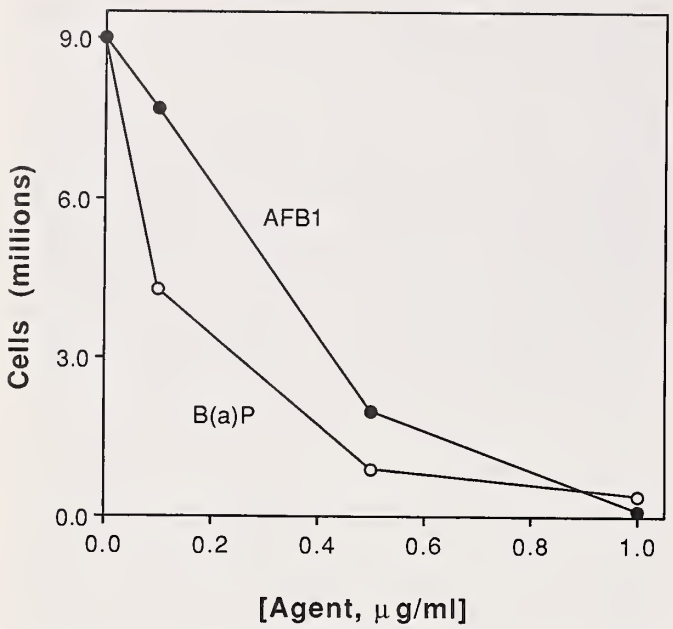

Fig. 3. Concentration dependence of toxicity (inhibition of growth) in SCC-9 cells by benzo(a)pyrene (B(a)P) or aflatoxin B_1 (AFB1) in the absence of a 3T3 feeder layer.

resulting in large size differences among species (*11*), and that the minimal immunochemical cross-reactivity, even between human and prosimian involucrins, can be explained by major differences in the repeating peptide structure comprising a large majority of the protein sequence (*20*). However, the unusual solubility properties that involucrins from diverse species share has facilitated their detection not only from primates but also from rabbits, dogs, pigs, rats, sheep, cats, and others (*8,21*, this work). Because immunochemical cross-reactivity can be equivocal or lacking, properties such as amino acid composition or ability to serve as a transglutaminase substrate provide important confirmatory evidence of correct identification (*8*). These results indicate the feasibility of using involucrin as a differentiation marker in animal models of human disease.

To interpret the significance of perturbed expression of differentiation markers in neoplastic conditions, an understanding of the molecular basis of such perturbation is essential. It is clear that involucrin and TG_K expression are readily perturbed by neoplastic processes in epithelial cells not only of the skin but also of the lung (*22*). In the latter, as well as in dysplastic lesions of oral epithelium (*23*), uncoupling of differentiation markers can occur. Such uncoupling can be induced in cultured keratinocyte carcinoma lines by short-term treatment with physiologic agents or carcinogens (*24–26*). Thus, perturbation in expression of individual markers may reflect more global changes in cell regulation. One likely contributory factor is the disturbance of DNA methylation patterns, a prevalent phenomenon in tumor cells. Alteration of methylation by 5-azacytidine treatment has revealed critical regulatory features of muscle cell lineage (*27*) and could do so for other differentiation programs, including that for keratinocytes.

The interaction of cultured keratinocytes with polycyclic aromatic hydrocarbons has been examined by numerous laboratories. Consistent with bioactivation of these mutagens (*4*), treated cultures exhibit induction of aryl hydrocarbon hydroxylase and obvious toxic effects (*17,28*). Present results point to potentially important interactions of agents that induce the biotransformation machinery with those that may not be good inducers themselves but are activated to potent toxins on metabolism. Aflatoxin B_1 is a potent animal carcinogen and a suspect carcinogen in human lung as well as liver (cf, *29*). Thus, exposure of responsive lung cells (including those exhibiting keratinocyte character) to aryl hydrocarbon hydroxylase inducers (such as TCDD or tobacco smoke) could potentiate the carcinogenic action of certain mycotoxins or other environmental agents. This general possibility has also been suggested for pyrolysis products in food that are activated by aryl hydrocarbon hydroxylase (*30*).

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Control of Squamous Differentiation in Tracheobronchial and Epidermal Epithelial Cells: Role of Retinoids

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ABSTRACT—Hyperplasia and squamous differentiation in epidermal and tracheobronchial epithelial cells is a multistage process. In stage I, quiescent progenitor cells are recruited to reenter the cell cycle. Protein kinase C activators, retinoids, cytokines, and polypeptide growth factors have been identified to control this stage of hyperproliferation. In stage II, cells become committed to irreversible growth arrest, which in normal cells appears to be a prerequisite for the expression of the differentiated phenotype (stage III). Confluence or treatment with interferon γ or phorbol esters are conditions that induce irreversible growth arrest and differentiation. Retinoids do not block stage II but specifically suppress the expression of stage III. The action of retinoids appears to be mediated by nuclear retinoic acid receptors. Studies understanding the mechanisms that regulate hyperplasia and squamous metaplasia may provide insight into the processes that lead to squamous cell carcinomas. Such studies may also provide new strategies for chemotherapy and chemoprevention. [J Natl Cancer Inst Monogr 13:93–100, 1992]

Epithelial cells from many tissues can undergo squamous differentiation. In certain tissues, as in skin and esophagus, this differentiation constitutes the normal pathway of differentiation whereas in other tissues, including the epithelium of the major conducting airways, it occurs only under pathological conditions (*1–3*). For example, during vitamin A deficiency and toxic or mechanical injury the normal pseudostratified, columnar tracheobronchial epithelium is replaced by a stratified squamous epithelium (*4–7*). This is exemplified in humans that by the numerous squamous metaplastic foci can be observed in the airways of heavy smokers (*8*). Although certain tissues, such as the esophageal epithelium, do not undergo cornification, squamous differentiation in various tissues exhibit many structural and biochemical similarities.

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HYPERPLASIA

The epidermal and tracheobronchial epithelium are in a continuous process of renewing themselves (*9–11*). In this process, terminally differentiated cells exfoliate and are replaced through the proliferation and subsequent differentiation of a distinct population of progenitor cells. To maintain the normal structure of the epithelium, the rate of proliferation and differentiation must equal the rate of cell loss. To establish such a balance, it is essential that proliferation and differentiation be regulated in a coordinate fashion. Such controls probably involve an equilibrium between factors that either promote or inhibit growth and differentiation. Flow cytometric analysis has indicated that in the normal skin a large percentage of the (stem) cells are in the resting (G_0) phase of the cell cycle (*12*). In response to wounding or exposure to phorbol esters or in certain hyperplastic skin disorders such as psoriasis, these quiescent cells are recruited to reenter the cell cycle resulting in hyperplasia of the epidermis (*13,14*). Similarly, hyperplasia of the tracheobronchial epithelium caused by vitamin A deficiency or by toxic or mechanical injury very likely involves the recruitment of the tracheobronchial progenitor (basal) cells (*4–7*).

A number of factors have been identified that regulate the proliferation and differentiation of epidermal and tracheobronchial epithelial cells in vitro and in vivo. These factors include epidermal growth factor (EGF), transforming growth factor-α (TGF-α), insulin, insulin-like growth factor I (IGFI), transforming growth factor-β (TGF-β), keratinocyte growth factor (KGF), interferon γ, certain interleukins, activators of protein kinase C, and retinoids (*15–21*; Jetten AM, unpublished observations). It is likely that in some instances the induction of the hyperproliferative response is mediated either through an increase in the synthesis or activation of certain polypeptide growth factors (Fig. 1). Such an hypothesis is supported by studies implicating increased synthesis of TGF-α as one of the factors promoting hyperplasia in psoriasis (*22*). In addition, administration of exogenous EGF has been reported to cause hyperplasia of tracheobronchial epithelial cells in fetal lambs (*23*).

REVERSIBLE AND IRREVERSIBLE GROWTH ARREST

Although our insight into the mechanisms that promote cell proliferation has increased in recent years, much less is

known about the mechanisms that negatively regulate cell proliferation. Tracheobronchial and epidermal keratinocytes can undergo several types of growth arrest. In normal skin and possibly in the tracheobronchial epithelium, many of the basal cells appear to be reversibly growth-arrested in the G_0 phase of the cell cycle (*12*). In culture, epidermal keratinocytes cells can be reversibly growth arrested by TGF-β (*20,21*). This growth arrest is accompanied by a reduction in c-*myc* expression (*24*). In tracheobronchial epithelial cells, TGF-β induces growth arrest and squamous differentiation at low density, while at high density it causes reversible growth arrest (*18,19*). Recently it has been reported that in mink lung epithelial MvLu-1 cells, TGF-β1 causes growth arrest in the late G_1 phase of the cell cycle (*25*). This growth arrest is associated with a decrease in the phosphorylation of the $p34^{cdc2}$ kinase as well as in its kinase activity.

Irreversible growth arrest in epidermal and tracheobronchial keratinocytes occurs when cells become committed to terminal differentiation. In vivo this happens when the basal cell moves from the basal to the suprabasal layers either of the epidermis or of the squamous metaplastic tracheobronchial epithelium. In vitro, epidermal and tracheobronchial epithelial cells undergo irreversible growth arrest when cultures reach the confluent stage of the growth curve (*2*) (Fig. 1). Although the molecular mechanisms controlling this commitment to terminal differentiation have yet to be established, changes in cell to cell interactions, in cell to substratum interactions, or in synthesis of differentiation factors are possibly involved. Addition of phorbol esters or interferon γ to epidermal or tracheobronchial epithelial cells growing in the exponential phase also induces irreversible growth arrest (*2,26,27*; Jetten AM, unpublished observations) (Fig. 1). In tracheobronchial epithelial cells, this can also be induced by the addition of diacylglycerols (*26*). The induction of terminal differentiation by phorbol esters and diacylglycerols is probably mediated by one of the protein kinase C isozymes. Bryostatins, macrocyclic lactones isolated from the marine bryozoan *Bugula neritina*, prevent the commitment to irreversible growth arrest induced by phorbol esters but not the growth arrest induced by interferon γ or confluence (*26,27*); Jetten AM, unpublished observations). These findings suggest that irreversible growth arrest induced by these three conditions is mediated by different signal transduction pathways. Senescence is another process of irreversible growth arrest that cells undergo after a few passages in culture. Senescent epidermal and tracheobronchial epithelial cells not only become irreversibly growth-arrested but also begin to express several squamous cell markers.

The molecular mechanisms that underlie irreversible growth arrest in epidermal and tracheobronchial epithelial cells have yet to be elucidated. Growth arrest induced by the different conditions may occur at different points in the cell cycle and only specific growth-arrest points may allow terminal differentiation whereas others do not (*28*). Specific alterations in gene expression during squamous differentiation are likely to be involved in controlling irreversible growth arrest. Such genes may fulfill a role similar to that of MyoD in myogenesis. MyoD belongs to a family of transcription factors that contain a basic region and an adjacent helix-loop-helix motif (*29*). Expression of this gene has been shown to cause growth arrest in several cell types. Furthermore, in many cell types the expression of MyoD induces expression of muscle cell-specific genes (*29,30*). The CCAAT-enhancer binding protein (C/EBP) is another example (*31*). The expression of this transcription factor causes growth arrest in fibroblasts and can induce adipocyte-specific gene expression in these cells. We propose that an induction of a specific transcription factor(s)—yet-unidentified growth arrest genes (GAGs)—

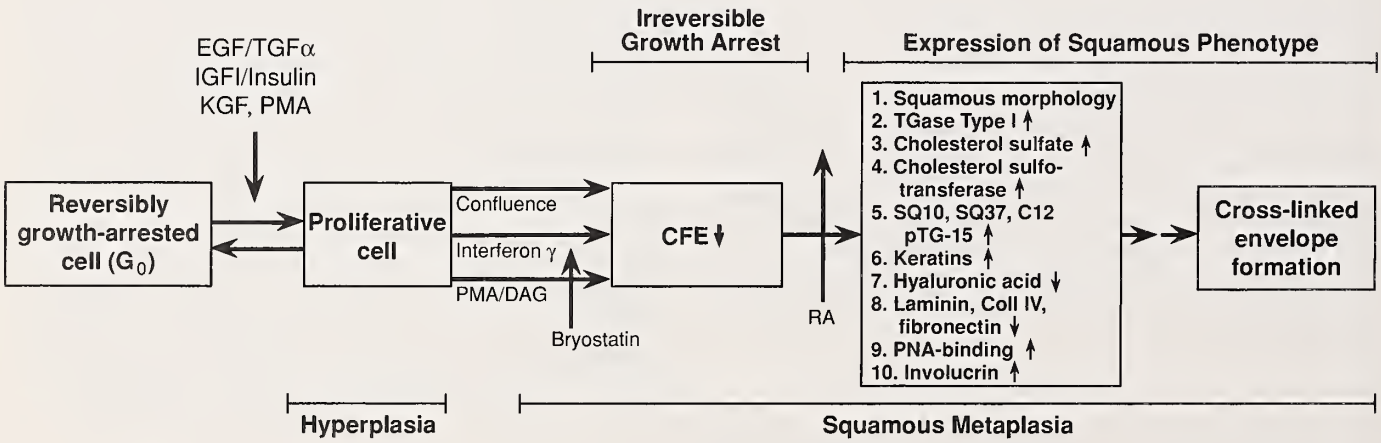

Fig 1. Schematic presentation of the multistage process of hyperplasia and squamous cell differentiation. First stage: quiescent progenitor cells are induced to proliferate by positive growth factors (e.g., TGFα, IGFI, KGF). This stage is also regulated by retinoids. Second stage: cells undergo irreversible growth arrest caused by confluence or treatment with phorbol esters, diacylglycerol (DAG), or interferon γ. Bryostatins prevent the induction of irreversible growth arrest by phorbol esters and DAG. Retinoids do not block this stage. Third stage: induction of the expression of the differentiated phenotype. Retinoic acid suppresses the induction of squamous cell-specific genes. In the final stage, cells undergo cornification.

may be instrumental in causing irreversible growth arrest in epidermal and tracheobronchial epithelial cells. As has been demonstrated for MyoD and C/EBP, these putative GAGs may, in addition to their effect on growth, also regulate the transcription of genes that encode markers of the squamous differentiated phenotype. This may occur in a direct manner through the interaction of the GAG protein with an enhancer element in the promoter region of squamous cell-specific genes. Alternatively, the putative GAG genes may act indirectly through the induction of other transcriptional factors (referred to here as DIF genes), which are not involved in controlling growth arrest but in solely regulating the transcription of squamous cell markers. A schematic presentation of this hypothetical model is shown in Fig. 2.

These putative GAG genes may be relevant to tumorigenesis and may function as tumor suppressor genes: loss of the expression of these genes may result in the inability of affected cells to undergo terminal differentiation.

EXPRESSION OF DIFFERENTIATED PHENOTYPE

Irreversible growth arrest appears to be a prerequisite for the induction of the squamous-differentiated phenotype in epidermal and tracheobronchial epithelial cells (*2*) (Fig. 1). A large number of biochemical and molecular squamous cell markers have been identified. The regulation of many of these squamous differentiation markers appears to be controlled at the transcriptional level (*2,32*). In addition, the onset of their expression occurs at different times during the differentiation process. The latter suggests that these genes are regulated by different mechanisms. One of the features of squamous cell differentiation in epidermal and tracheobronchial epithelial cells is the formation of the cross-linked envelope, a layer of cross-linked protein deposited beneath the plasma membrane. Transglutaminase has been identified as the enzyme

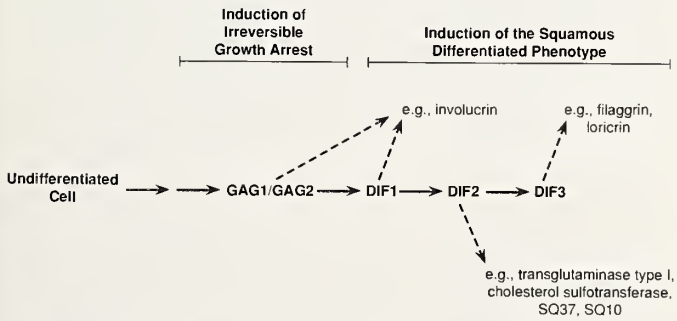

Fig. 2. Hypothetical model of the induction of squamous differentiation. Induction of a unique set of growth arrest genes (e.g., GAG1/GAG2) causes irreversible growth arrest in epidermal or tracheobronchial epithelial cells. These genes may fullfil a role similar to that of MyoD or C/EBP in myogenesis or adipocyte differentiation, respectively. They may induce the expression of certain squamous cell-specific genes directly or through the induction of putative DIF genes. DIF transcriptional factors do not affect cell growth but regulate solely the expression of a specific set of squamous cell-specific genes.

that catalyzes the formation of the interpeptide bonds between protein precursors of the cross-linked envelope. The epidermal or type I transglutaminase is present in the suprabasal layers of the epidermis and in squamous metaplastic foci of the tracheobronchial epithelium (*33,34*). In cultured epidermal and tracheobronchial epithelial cells transglutaminase type I is increased when cultures reach confluence or when logarithmic cultures are treated with phorbol esters or interferon γ (*26,27,35*; Jetten AM, unpublished observations). The type I transglutaminase from rabbit, human, and rat has been recently cloned and sequenced (*36,37*). The deduced amino acid sequences of these three transglutaminases are about 92% identical, indicative of the conservation of important structural features. The induction of transglutaminase type I activity is related to an increase in transglutaminase protein and mRNA (*36*). Another transglutaminase (type E) has been identified in the skin that appears to be induced at later stages of differentiation (*38*). Involucrin (*32*) and loricrin (*39*) have been identified as protein precursors of the cross-linked envelope. It is probable that the formation of the cross-linked protein layer occurs in several stages in order to form an organized layer of cross-linked protein and not a randomly cross-linked aggregate of protein. Transglutaminase type I and involucrin may play a major role in the early stages of cross-linked envelope formation whereas transglutaminase E and loricrin may be important in later phases. Recently, lipocortin I has been found to be an excellent substrate for transglutaminase type I and II (*40*; Jetten AM and Moore G, unpublished observations). An antibody raised against cross-linked envelopes prepared from rabbit tracheal epithelial cells reacted specifically with a 36-kd protein that was identified as lipocortin I. A function for the cross-linking of lipocortin I has still to be established. In epidermal and tracheobronchial epithelial cells squamous differentiation is accompanied by a stimulation of cholesterol sulfate synthesis (*26,27,41*). This enhancement is due to an increase in the synthesis of cholesterol sulfotransferase (*42*).

Although epithelial cells from different tissues follow largely a similar program of squamous differentiation, these programs are not identical. For example, the induction of squamous differentiation in epidermal keratinocytes is accompanied by an increase in the expression of keratins 1 and 10 (*32,43*) whereas in tracheobronchial epithelial cells squamous differentiation is accompanied by an increase in the expression of keratin 13 (*44*). These alterations in keratin expression occur relatively early in the differentiation process.

Several changes in cell surface properties have been reported to accompany squamous differentiation. Glycoproteins isolated from undifferentiated epidermal and tracheobronchial epithelial cells are unable to bind the lectin peanut agglutinin (PNA) whereas squamous-differentiated cells contain glycoproteins that are able to bind PNA (*45*; Jetten AM, Lotan R, unpublished observations). Undifferentiated cells express relatively high levels of fibronectin, laminin B1, and collagen IV mRNA; how-

ever, during squamous differentiation these mRNA levels are dramatically reduced (*46*). Extracellular matrix components have been reported to influence the migration and differentiation of human epidermal keratinocytes. Fibronectin, in particular, has been shown to increase migration and to inhibit squamous differentiation. Treatment of epidermal keratinocytes with TGF-β1 enhances the synthesis of fibronectin, collagen IV, and laminin (*46*). The inhibition of squamous differentiation by TGF-β may at least in part be related to this increase in fibronectin synthesis.

ACTION OF RETINOIDS

Retinoids have multiple and sometimes contrasting effects on the skin. For example, application of retinoids onto normal skin causes increased proliferation whereas retinoids reverse hyperplasia in psoriatic skin. In vitro and in vivo studies have indicated that a critical level of retinoic acid is required for the maintenance of the normal structure of the skin (*47*). Hypovitaminosis leads to excessive differentiation (hyperkeratosis) whereas hypervitaminosis results in reduced maturation (parakeratosis). In cultured epidermal keratinocytes, retinoids have been shown to suppress the expression of several squamous cell markers (*2,27,32,48*). Retinoids also play an important role in the maintenance of the normal epithelium of the major airways. For example, vitamin A deficiency in animals has been shown to cause hyperplasia and squamous metaplasia of the tracheobronchial epithelium (*2,4,6*). This condition can be reversed by vitamin A supplementation. A higher intake of retinoids is able to convert many of the squamous metaplastic regions in the lungs of heavy smokers to a normal epithelium (*8*). EGF-induced hyperplasia of the tracheobronchial epithelium in fetal lambs has also been reported to be suppressed by retinoic acid (*23*). In vitro studies using explant or cell culture systems have confirmed the central role that retinoids play in determining whether the tracheobronchial epithelium undergoes a pathway of normal mucociliary differentiation or squamous cell differentiation (Fig. 3). Tracheobronchial epithelial cells grown in the absence of retinoids start to express many biochemical and molecular markers of squamous differentiation including involucrin, transglutaminase type I, cholesterol sulfate, and keratin 13 (*2,35,41,42,44*). The presence of retinoids induces the formation of columnar cells that contain tight junctions and secretory granules and that secrete mucin-like glycoconjugates (*34*).

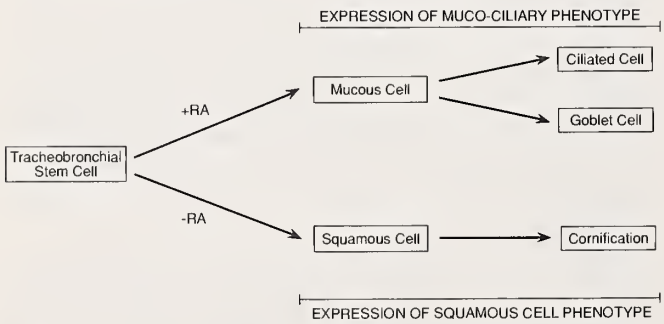

Fig. 3. Central role of retinoic acid in the regulation of differentiation of tracheobronchial progenitors. Absence of retinoids induces a pathway of squamous differentiation whereas the presence of retinoids induces a pathway of mucociliary differentiation. The induction of RARβ by retinoic acid may be a prerequisite for the induction of mucociliary differentiation.

The suppression of squamous cell markers occurs at nanomolar concentrations of retinoic acid (*2,32,35,44*). Moreover, a specific chemical structure is critical to the ability of retinoids to inhibit squamous differentiation (*35, 49*). These observations suggest that specific retinoid receptors mediate the action of retinoids in epidermal and tracheobronchial epithelial cells.

RETINOIC ACID RECEPTORS AND BINDING PROTEINS

Several cytosolic retinoid binding proteins and nuclear retinoic acid receptors have been identified in eukaryotic cells. The nuclear retinoic acid receptors, RARα, RARβ, and RARγ (*50–54*), encode one group of retinoic acid-dependent transcription factors. The RARs display a structural organization very similar to that of the family of steroid/thyroid hormone receptors (*55*). The RARα, RARβ, and RARγ receptors exhibit a high degree of homology between their DNA- and ligand-binding domains whereas little homology exists between their A and F domains. It has been reported that each of the RAR genes can give rise to more than one transcript via alternative splicing (*56,57*). These transcriptions differ in their 5′-regions. Differences in the A- and F-domains of the different RAR proteins may be important in specifying their interaction with other nuclear proteins. Recently, another group of retinoid-dependent transcription factors, named RXRs, was identified (*73*). The ligand binding domain of the RXRs exhibits little homology with that of the RARs suggesting differences in ligand binding specificity between RARs and RXRs. This was demonstrated by studies showing that RXRα does not blind all-*trans*-retinoic acid very effectively but is able to bind 9-*cis*-retinoic acid with high affinity (*74,75*).

Studies using in situ hybridization have indicated that RARα and RARγ transcripts are present in the skin whereas RARβ transcripts are absent (*58–60*). The latter is in contrast to the tracheobronchial epithelium in which the RARβ gene is highly expressed (*59*). Tracheobronchial and epidermal keratinocytes in culture both express RARα and RARγ transcripts (*61*; Vollberg TM, et al, unpublished observations). Retinoic acid treatment induces the expression of RARβ transcripts in tracheobronchial epithelial cells but not in epidermal keratinocytes. In tracheobronchial epithelial cells this induction of RARβ coincides with the suppression of squamous cell markers (*61*). Since retinoic acid does not include RARβ in epidermal

keratinocytes, we believe that RARβ is not involved in the suppresion of squamous differentiation but in the regulation of the normal mucociliary phenotype of the tracheobronchial epithelium (Fig. 3).

Retinoids have been shown to be able to up- and down-regulate gene expression. The classic model of hormone action is one mechanism by which retinoids can enhance transcription. According to this model, binding of a retinoid to RARs or RXRs stimulates the formation of homodimeric or heterodimeric complexes, which then interact with specific retinoid-response elements (e.g., RARE or RXRE) resulting in an alteration of the rate of transcription of target genes. Recently, RXRβ has been shown to form heterodimers with the RAR, thyroid hormone, and vitamin D receptors, thereby enhancing both their DNA binding and transcriptional activation through their cognate response elements (*76*). RAREs have been identified in the promoter region of several genes, including the RARβ gene (*62*). These RAREs are composed of a direct repeat of the sequence of A/GGTTCA separated by five nucleotides. Recently, an RXRE, consisting of a tandem repeat of the sequence AGGTCA spaced by one nucleotide, was found in the promoter region of the cellular retinol binding protein type II (*77*).

Negative regulation of gene expression may occur through several different mechanisms. Three major possibilities are shown in Fig. 4. The first mechanism is an example of the classic repressor model. The nuclear retinoic acid receptor-retinoic acid complex (RAR-RA) may bind to the identical enhancer element as the transcriptional activator X, thereby competing for the same site and suppressing transcription. A variation of this model is that the RAR-RA complex blocks binding of X by interacting with a site close to or overlapping its enhancer. Squelching (*63*) is another mechanism of suppression of gene expression: the RAR-RA complex binds to a transcriptional activator X thereby preventing X from interacting with its enhancer element. The RAR-RA complex can also act by interfering with the activation of a bound transactivator, a mechanism known as quenching (*78*). A clear picture has yet to emerge on the mechanisms by which retinoic acid suppresses the induction of squamous cell markers such as keratins and transglutaminase type I. Retinoic acid could suppress the expression of squamous cell-specific genes by reducing their rate of transcription or mRNA stability, and both of these mechanisms could involve RARs and/or RXRs (*32,49,61*). Above, we have proposed that the increase in the transcription of squamous cell-specific genes requires the induction of specific transcriptional regulators, products of the putative GAG and DIF genes. Retinoids may suppress the expression of squamous cell markers by interfering with the action of GAG or DIF proteins by any of the three mechanisms just described. Alternatively, retinoids may suppress the transcription of GAG or DIF genes themselves. Since retinoids do not block irreversible growth arrest in epidermal and tracheobronchial epithelial cells, they may not alter the expression of GAGs.

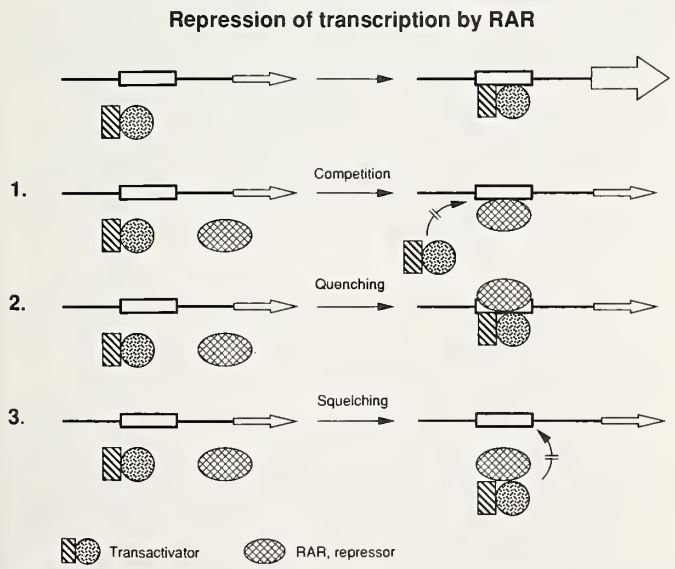

Fig. 4. Three major mechanisms of gene suppression by retinoic acid. 1. A homodimeric or heterodimeric complex of RAR-RA competes with a transcriptional activator X for the same site. 2. Quenching: RAR-RA complex binds to a bound transcriptional activator X thereby preventing transcriptional activation. 3. Squelching: RAR-RA complex binds to a transcriptional activator X thereby preventing X from binding to its enhancer.

Examination of RAR expression in several human lung carcinoma cell lines showed that these cells contained two RARα transcripts similar in size and amount to those in normal tracheobronchial cells (Table 1) (*61*). Several carcinoma cell lines exhibited an altered expression of RARβ and RARγ compared to that in normal tracheobronchial epithelial cells (Table 1). Since retinoic acid plays a central role in controlling proliferation and differentiation in the tracheobronchial epithelium, it is conceivable that RARs act as putative tumor suppressors or proto-oncogenes and are involved in lung carcinogenesis. A translocation between chromosome 15 and 17 in acute promyelocytic leukemia cells has been found to involve the first intron of RARα thereby implicating RARα in this neoplastic process (*64*). The loss of the p21-p25 region of chromosome 3 is common to all major types of lung cancer and is near the location of the RARβ gene (*65*). Restriction fragment analysis of DNA isolated from several lung carcinoma cell lines did not provide any evidence of rearrangements in the RAR genes (Nervi C, Jetten AM, unpublished observations). However, genetic changes other than deletions or translocations (e.g., mutations) could affect the function of a specific RAR and contribute to the neoplastic nature of affected lung epithelial cells.

Two types of cellular retinol binding proteins (CRBPI and CRBPII) and two types of cellular retinoic acid binding proteins (CRABPI and CRABPII) have been identified. They bind, respectively, retinol or retinoic acid and many of its derivatives (*66-68*). Tracheobronchial epithelial cells contain CRBP; however, little CRBP has been detected in epidermis (*59,69,70*). In vitro studies have indicated that the presence of CRBP stimulates the formation of retinyl esters by the endoplasmic reticulum suggesting a role for CRBP in the metabolism of retinol to esters

Table 1. Comparison of RAR expression in lung carcinoma cells

Histologic type	RARα		RARβ				RARγ	
			−RA		+RA			
Cell line	3.5	2.6	3.2	2.9	3.2	2.9	3.3	3.1
Normal HBE	+ +	+ +	−	−	+	+	−	+
Large cell carcinoma								
H460	+ +	+ +	+	+	+ +	+ +	−	−
Small cell carcinoma								
H69	+ +	+ +	+ +	−	+ + +	+ +	−	−
Adeno(squamous)carcinoma								
H125	+ +	+ +	−	−	−	−	+ + +	+
H647	+ +	+ + +	+	−	+	+	−	+ +
SK-LU-1	+ +	+ + +	+	+	+ +	+	+ +	−
H596	+ +	+ + +	−	−	−	−	+ +	−
A1188	+ +	+ +	−	−	−	−	+ +	−
KNS-62	+ +	+ +	+	−	+	−	+ +	−
A549	+ +	+ +	+	+ +	+	+ +	+	+
Squamous cell carcinoma								
Calu-1	+ +	+ +	−	−	+ +	+ +	−	+ +
H520	+ +	+ +	+	−	+	−	+	−
SK-MES-1	+ +	+ + +	−	−	−	−	−	+
Mucoepidermoid								
H292	+ +	+ +	+	+	+	+	+ +	+

and possibly dehydroretinol, retinal, and retinoic acid (*71*). Cultured tracheobronchial epithelial cells contain very low levels of CRABP-binding activity and CRABPII mRNA (Vollberg TM, Jetten AM, unpublished observations). Basal cells in the skin and undifferentiated epidermal keratinocytes in culture also express low levels of CRABPII protein and mRNA. When epidermal cells undergo squamous differentiation, the level of CRABPII greatly increases (*69*). Although a function for the CRABPs has yet to be established, a role for CRABP in facilitating retinoic acid metabolism has been proposed (*48*). In this manner CRABP may control the effective concentration of retinoic acid in the cell (*72*) or promote the formation of specific metabolites such as 9-*cis*-retinoic acid. The reported increase in the levels of CRABPII during squamous differentiation may result in reduced levels of retinoic acid and allow expression of squamous cell markers (*48*). Alternatively, CRABP may play a totally different role. For example, it may be involved in controlling the stability of specific mRNAs.

In recent years a lot of progress has been made understanding the regulation of hyperplasia and squamous metaplasia in epidermal and tracheobronchial epithelial cells and the role that retinoids play in these processes. These basic studies will help to provide a better insight into the processes that lead to neoplasia in these tissues and may provide new strategies for chemoprevention and chemotherapy.

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Suppression by Retinoic Acid of Epidermal Growth Factor Receptor Autophosphorylation and Glycosylation in Cultured Human Head and Neck Squamous Carcinoma Cells[1]

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ABSTRACT—The epidermal growth factor receptor (EGF-R) gene is overexpressed or amplified in various human squamous cell carcinomas, including those of the head and neck (HNSCC). Earlier we found that β-all-*trans*-retinoic acid (RA) inhibited the growth and suppressed the aberrant squamous cell differentiation of several cultured HNSCC cell lines. Here we examined the effects of RA on the expression and function of EGF-R in two HNSCC cell lines, 1483 and 183, which exhibit distinct states of squamous cell differentiation, EGF-R mRNA levels, and responses to the growth inhibitory effects of RA. Treatment with RA (1 μM, 7 days) of the RA-sensitive 1483 cells decreased the level of EGF-R mRNA two- to four-fold and the binding of ^{125}I-EGF to the cell surface by 30%–35%. In contrast, RA treatment of the 183 cells did not alter the EGF-R mRNA level or the binding of ^{125}I-EGF. Other effects of RA on EGF-R structure and function were similar in both cell lines. RA did not alter the amount of immunoprecipitable [^{35}S]methionine-labeled cellular EGF-R, ^{125}I-cell surface labeled EGF-R, EGF-R internalization, or transforming growth factor alpha (TGF-α) mRNA. More important, RA treatment of both cell lines decreased EGF-R autophosphorylation activity detected in immune-complex-kinase assay by about three- and five-fold in the 1483 and 183 cells, respectively. Likewise, RA decreased the glycosylation of EGF-R in both cell lines. In the 1483 cells, RA suppressed the incorporation of either glucosamine or fucose by about 50%, whereas in the 183 cells RA suppressed the incorporation of fucose by about 80%. These results demonstrate that RA can modify the structure of the EGF-R by decreasing its glycosylation and suggest that these changes may suppress the autophosphorylation activity of the receptor kinase. The RA-induced changes in EGF-R do not correlate with the effect of RA on the growth of the cells but may be related to the suppression of squamous cell differentiation in the 1483 cells. [J Natl Cancer Inst Monogr 13:101–110, 1992]

Epidermal growth factor receptor (EGF-R), a transmembrane glycosylated phosphoprotein with a molecular weight of about 170 kd (*1–3*), is the mediator of signal transduction for the mitogenic polypeptides epidermal growth factor (EGF) (*4,5*) and transforming growth factor-α (TGF-α) (*5,6*). Binding of these factors to the extracellular ligand-binding domain of EGF-R results in a rapid activation of an intrinsic protein tyrosine kinase located in the cytoplasmic C-terminus domain of the receptor (*3*). The activation of the EGF-R kinase results in both phosphorylation of tyrosine residues on the receptor itself and of several cellular proteins such as phospholipase C-γ1 (*1–3,7,8*). The kinase activity leads to other events implicated in signaling pathway, including an enhanced production of inositol 1,4,5-trisphosphate and diacylglycerol (*8*) as well as the internalization of the ligand-receptor complex (*1,2*).

The expression of EGF-R can be regulated at the transcriptional, posttranscriptional, and posttranslational levels in different cell types (*9*). Many epidermoid carcinomas, including head and neck squamous cell carcinomas (HNSCC), overexpress the EGF-R in vivo (*10–13*) and in culture (*14–18*). Some HNSCC also produce TGF-α (*19–21*). Overexpression of EGF-R (*22*) in combination with expression of EGF or TGF-α (*6*) in immortalized cells has led to malignant transformation resulting from an autocrine growth stimulation loop (*6,22*). Furthermore, the development of tumors in an animal model for oral carcinogenesis is associated with an increased expression of EGF-R (*23–25*) and TGF-α (*26*). Therefore, it was proposed that the ability of tumor cells to produce and secrete TGF-α and to overexpress EGF-R on their surface may provide them with an autonomous growth that is regulated by an autocrine growth stimulation loop (*5,20,21,27*).

Several studies have shown that vitamin A and some of its metabolites and synthetic analogues (retinoids) are effective inhibitors of dimethylbenz[*a*]anthracene (DMBA)-induced carcinogenesis in the hamster buccal and lingual mucosa (*28–30*). Furthermore, oral leukoplakia lesions in

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humans responded to 13-*cis* retinoic acid (13-CRA) treatment with regression of the dysplastic changes and regaining of an apparently normal histology (*31*), and the development of second primary tumors was suppressed in HNSCC patients treated with 13-CRA after surgery and radiation therapy of the first primary tumor (*32*).

Vitamin A and some retinoids have been shown to modulate the growth and differentiation of various premalignant and malignant cells in vivo and in culture (*33–36*). In previous studies, we demonstrated the ability of β-all-*trans*-retinoic acid (RA) to inhibit the growth of seven of eight HNSCC cell lines in monolayer culture and to prevent three HNSCC cell lines from forming colonies in semisolid agarose (*37–39*). Furthermore, RA suppressed the aberrant squamous cell differentiation of several HNSCC cell lines (*38–41*). As a part of an ongoing investigation of the mechanism by which RA modulates the growth and differentiation of HNSCC cells we have addressed the effect of RA on expression, structure, and activity of EGF-R. Numerous reports have demonstrated the ability of retinoids to modulate the binding of EGF to the surface of a variety of cells (*34,42–47*) and to alter EGF-R gene expression (*45–52*). Furthermore, our previous studies have shown that RA decreased the tyrosine protein kinase activity of EGF-R in human glioblastoma cells (*53,54*), and others have reported that RA decreased the expression of TGF-α in human embryonal carcinoma cells (*55*).

In previous investigations we demonstrated that RA suppressed the growth and proliferation of the 1483 HNSCC cells but had only a limited effect on the growth of the 183 HNSCC cells (*37,39*). The levels of EGF-R mRNA (*11*), protein, and basal tyrosine protein kinase activity (*56*) were reported to be higher in the 1483 cells than in the 183 cells. Because of the differences in EGF-R expression, the 1483 and 183 HNSCC cell lines were chosen for the present study, which examined the effect of RA on the expression of EGF-R and TGF-α mRNAs and on the function of EGF-R, to determine whether the differential responsiveness of these two cell lines to RA resulted from differences in modulation of EGF-R.

MATERIALS AND METHODS

Cells and Culture Procedures

The HNSCC cell lines 1483 and 183, described previously (*37,57*), were obtained from Dr. Peter Sacks (The University of Texas M.D. Anderson Cancer Center, Houston, Tex). Line 1483 was derived from a 66-year-old male diagnosed as having a $T_2N_1M_0$ well-differentiated squamous carcinoma of the retromolar trigone. Line 183 was derived from a 54-year-old male diagnosed as having a $T_3N_0M_0$ well-differentiated squamous carcinoma of the tonsil. The A431 cells (ATCC CRL 1555), which were derived from a human epidermoid carcinoma of the vulva of an 85-year-old female (*58*) and overexpress the EGF-R gene (*14*), were obtained from the American Type Culture Collection (Rockville, Md.). The cells lines were propagated in a 1:1 (vol/vol) mixture of Dulbecco's modified Eagle's minimum essential medium (DMEM) and Ham's F12 medium supplemented with 10% fetal bovine serum, penicillin, and streptomycin. The cells were grown in 10-cm diameter tissue culture dishes placed in a humidified atmosphere consisting of 5% CO_2 and 95% air in an incubator maintained at 37 °C. For biochemical assays, the cells were washed twice with precooled Ca^{2+}-free and Mg^{2+}-free phosphate-buffered saline, pH 7.2, and harvested by gentle mechanical scraping.

Treatment of Cells with RA

The RA was obtained as a gift from BASF (Ludwigshafen, Germany). A stock solution of 10 m*M* RA was prepared in dimethyl sulfoxide (DMSO) and diluted into growth medium to obtain final concentrations between 0.01 and 10 μ*M*. Control cultures received medium with 0.01 or 0.1% DMSO alone. Cells were treated for 7 days, and the culture medium was replaced every 72 hours with fresh medium, supplemented with either RA or DMSO. To prevent cells from reaching a high density, the cells were detached after 3 days of culture and replated at a low density and refed with fresh medium supplemented with RA or DMSO. Under such conditions the cultures were about 70% confluent after a total of 7 days of growth.

Analysis of TGF-α and EGF-R mRNAs

RNA samples were prepared using the guanidine isothiocyanate/cesium chloride method. Quantitative assessment of mRNA expression was performed using the RNA dot-blot and/or Northern blot analysis as described by Untawale and Blick (*59*). In brief, for the dot-blot analysis, 50 μg of total cellular RNA was serially diluted (1:1) in an SSC solution (12× sodium chloride and 8× sodium citrate) containing 12.8% formaldehyde and blotted onto nylon filters. For the Northern blot analysis, poly(A)$^+$ RNA was isolated using oligo(dT)-cellulose chromatography. mRNA (10 μg/lane) was electrophoresed in formaldehyde/1% agarose gel and blotted onto a nylon filter. The filters were incubated with ^{32}P-labeled cDNA probes, washed, and placed against an x-ray film for autoradiography. The probes used were HER-A64-1 (*11,14*) for EGF-R, and pTGF-C1 (*60*) for TGF-α. The same filters were rehybridized with a chicken β-actin cDNA probe to standardize mRNA loading. The autoradiograms were scanned using a spectrophotometer (Beckman Instruments, Fullerton, Calif.), and the data were used for quantitation of the amounts of the mRNAs.

Binding of ^{125}I-Labeled EGF to Cells

Receptor-grade EGF and ^{125}I-labeled EGF were purchased from Collaborative Research (Boston, Mass.). Binding assays were performed according to procedures previously described (*54*). Briefly, cells that had been grown for 5 days in the presence of either 1 μ*M* RA or DMSO were detached and replated in 12-well plates (0.5 or 1 × 10^5 cells/well) and cultured for 2 days in medium

containing 10% serum and supplemented with RA or DMSO. The medium was then removed by aspiration, and the cells were washed once with DMEM:F12 and incubated for an additional 24 hours in serum-free DMEM:F12 with RA or DMSO. The dishes were then washed twice in cold Dulbecco's phosphate-buffered saline (DPBS) containing 0.2% bovine serum albumin (BSA). To each well was added 1 mL of ^{125}I-labeled EGF at various concentrations in the range from 1 to 35 ng/mL in DPBS:BSA solution, and the plates were incubated for 2 hours at 4 °C. The cells were then washed five times with DPBS:BSA and lysed with 1% sodium dodecylsulfate (SDS) in 0.1 *M* NaOH. The radioactivity was measured using a Beckman 5000 gamma counter. Nonspecific binding was determined from the amount of radioactivity bound in the presence of a 100-fold excess of unlabeled EGF and was usually less than 4% of the total binding. The number of EGF binding sites and the dissociation constants (K_d) were determined according to Scatchard's (*61*) method using a linear regression analysis.

Immunoprecipitation of EGF-R

Cells were metabolically radiolabeled by incubating the cultures with methionine-free medium and dialyzed fetal bovine serum for 2 hours and then overnight with 70 μCi/mL [^{35}S]methionine (ICN, Irvine, Calif.) or by incubating the cultures for the last 48 hours of growth with either [^{3}H]glucosamine or [^{3}H]fucose, both at 40 μCi/mL. Some cultures were subjected to cell surface ^{125}I-iodination using the lactoperoxidase-catalyzed iodination method described elsewhere (*62*). The radiolabeled cells were washed twice with cold DPBS and homogenized in a lysis buffer (1% Triton X-100, 150 m*M* NaCl, 5 m*M* EDTA, 4 m*M* phenylmethylsulfonyl fluoride [PMSF], 1% aprotinin in 20 m*M* sodium phosphate, pH 7.5). The homogenates were cleared by centrifugation for 10 minutes at 10 000*g*, and the supernatant fraction was adjusted to 250 μg protein/mL. Retinoic acid treatment did not alter the amount of protein per cell; therefore, lysates of untreated and treated cells containing the same amount of protein represented similar cell numbers. The lysates were then preadsorbed by adding Pansorbin (50 μL of a 10% wt/vol suspension per mL lysate) and recentrifugation. Purified R1 monoclonal anti-EGF-receptor antibody (Amersham Corp., Arlington Heights, Ill.) was added at 0.5 μg/mL lysate, and the lysates were incubated on ice. After 1 hour, 50 μL of Pansorbin was added to the lysates, and they were incubated for an additional 20 minutes at 4 °C. The immune complex, which precipitated with the Pansorbin, was washed three times with a mixture of 0.1% Triton X-100 and 150 m*M* NaCl in 10 m*M* sodium phosphate, pH 7.5, and was collected by centrifugation (10 000*g*, 2 minutes). The immunoprecipitates were subjected to electrophoresis in 6% polyacrylamide slab gels in the presence of SDS (SDS-PAGE), as described elsewhere (*62*). Gels containing ^{35}S-labeled samples were impregnated with En^{3}Hance (New England Nuclear, Boston, Mass.) and then were dried, placed against an x-ray film, and kept at −70 °C for fluorography.

Autophosphorylation of Immunoprecipitated EGF-R

The cell extracts were prepared as previously described. The R1 antibody (4 μL) was added to the lysate, and the mixture was incubated for 1 hour at 4 °C, followed by addition of 50 μL of Pansorbin and another 20-minute incubation. The immune complex was washed three times as previously described. The phosphorylation reaction was initiated by adding 50 μL of 0.01 μCi of γ-[^{32}P]adenosine triphosphate (ATP), 2000–3000 Ci/mmol (New England Nuclear) and 20 m*M* $MnCl_2$ in 20 m*M* HEPES, pH 7.0, to the immunoprecipitated pellet. After 10 minutes at room temperature, the reaction was terminated by the addition of 1 mL of lysis buffer. The precipitates were washed again and then subjected to SDS-PAGE analysis.

Analysis of EGF-R Internalization

Internalization of cell-surface-bound ^{125}I-labeled EGF was analyzed as described previously (*54*). Briefly, the cells were incubated with ^{125}I-labeled EGF for 45 minutes at 4 °C, washed three times in cold DPBS containing 0.2% BSA, and brought to 37 °C. After various times, triplicate cultures were washed three times with ice-cold DPBS containing 0.2% BSA and treated with 0.2 *M* acetic acid in 0.5 *M* NaCl for 6 minutes at 4 °C to remove cell-surface-bound ^{125}I-labeled EGF. The cells were then lysed with 1% SDS in 50 m*M* NaOH, and the radioactivity was measured as described previously. The percentage of internalized EGF was determined from the ratio between the radioactivity associated with the cells after acid treatment and the total cell-associated radioactivity at time 0 before acid treatment (*54*).

RESULTS

Effects of RA on EGF-R and TGF-α mRNA Levels

The 1483 HNSCC cells contained eight to sixteen times higher levels of EGF-R mRNA than did the 183 cells (Fig. 1), in agreement with a previous report (*11*). However, the two cell lines expressed similar levels of TGF-α mRNA (Fig. 1) as reported for other HNSCC cell lines (*23,24*). Treatment of the 1483 cells with RA decreased their levels of EGF-R mRNA two- to four-fold, as evidenced by both dot-blot and Northern blot analyses (Fig. 1). In contrast, RA did not alter the level of EGF-R mRNA in the 183 cells. Furthermore, RA did not alter the level of TGF-α mRNA in either of the two HNSCC cell lines (Fig. 1).

Effects of RA on the Binding of ^{125}I-Labeled EGF to the Surface of HNSCC Cells

The binding of ^{125}I-labeled EGF to the surface of untreated 1483 HNSCC cells at saturation was about twice as high as the binding to the surface of the 183 cells (Fig. 2, *upper panels*). Treatment with RA decreased the binding of ^{125}I-labeled EGF to the 1483 cells (0.8×10^6 receptors/cell) by about 25% compared to untreated con-

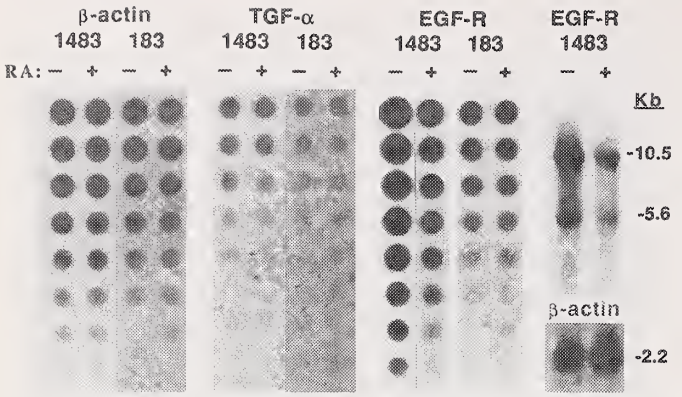

Fig. 1. Expression of EGF-R and TGF-α in untreated and RA-treated human 1483 and 183 HNSCC cells. Cells were grown for 7 days in medium supplemented with 0.01% DMSO (−) or 1 μ*M* RA (+), and the total RNA was isolated and used for dot-blot analysis of the levels of the mRNAs for EGF-R, TGF-α, and β-actin. Poly(A)$^+$RNA was isolated from the 1483 cells and analyzed (10 μg/lane) for the expression of EGF-R and β-actin mRNA by Northern blotting.

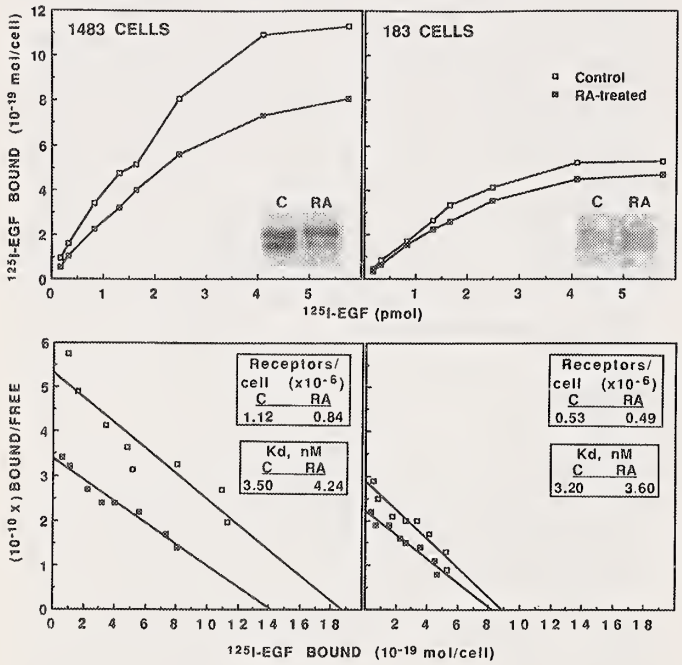

Fig. 2. Analysis of the binding of ^{125}I-labeled EGF to untreated and RA-treated human 1483 and 183 HNSCC cells. *Upper panels:* cells were grown for 7 days in medium supplemented with 0.01% DMSO (control, or C) or 1 μ*M* RA (RA-treated, or RA), and the binding of increasing concentrations of ^{125}I-labeled EGF to their surface was analyzed as described in Materials and Methods. *Insets*, autoradiograms of EGF-R immunoprecipitated from lysates of cell-surface-^{125}I-iodinated cells. *Lower panels:* Data from the saturation binding curves were analyzed according to Scatchard (*61*) to determine the number of receptors/cell and the K_d (insets).

trols (1.1×10^6 receptors/cell) and increased the apparent K_d by about 20% (Fig. 2). In contrast, the binding of EGF to the 183 cells was not affected significantly by RA treatment (Fig. 2). The K_d values ranged between 3.2 and 4.2, indicating that most of the receptors are of the low affinity type. Immunoprecipitation of cell surface ^{125}I-iodinated EGF-R revealed the presence of the intact 170-kd EGF-R and a 150-kd protein that is most likely a partially degraded receptor. The amount of labeled EGF-R was similar in untreated and RA-treated cells, indicating that similar amounts of EGF-R were exposed on the surface of untreated and RA-treated 1483 or 183 cells and that the amount of cell surface EGF-R was higher in the 1483 than in the 183 cells (Fig. 2, *inserts in upper panels*).

Effect of RA on the Amount of EGF-R Protein in HNSCC Cells

Some of the results described previously suggested that RA treatment of the 1483 cells decreased the expression of EGF-R. To further examine the effect of RA, the steady state level of EGF-R protein was analyzed by metabolic labeling of the cells with [^{35}S]methionine, followed by cell solubilization and immunoprecipitation of [^{35}S]methionine-labeled EGF-R with anti-EGF-R antibody R1, followed by SDS-PAGE and fluorography. This analysis (Fig. 3, *lower panel*) revealed that the amount of EGF-R

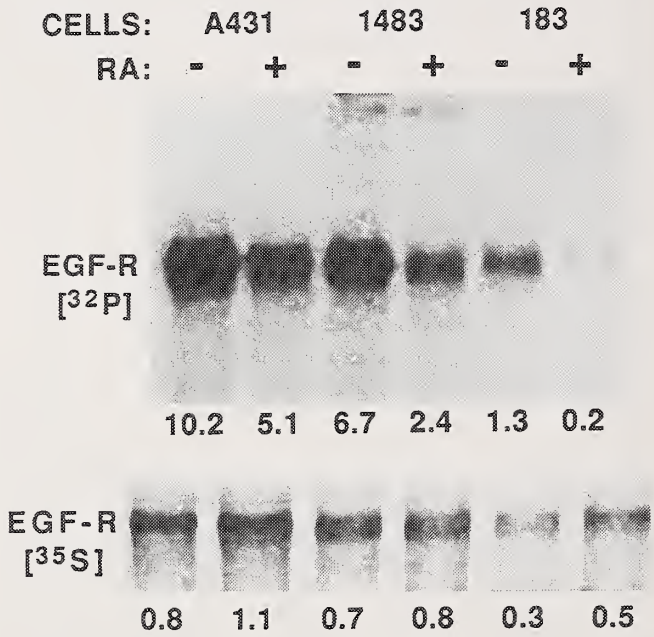

Fig. 3. Suppression of EGF-R autophosphorylation in squamous carcinoma cells by RA. A431, 1483, and 183 cells were grown for 7 days in medium supplemented with 0.01% DMSO (−) or 1 μ*M* RA (+), and some cultures were labeled with [^{35}S]methionine (70 μCi/mL) for the last 12 hours. Cells were then harvested and extracted, and the EGF-R was immunopreeipitated using anti-EGF-R antibodies as described in Materials and Methods. Immunoprecipitates were analyzed by the immune-complex-kinase assay followed by SDS-PAGE and autoradiography to determine the protein kinase activity of the EGF-R (*upper panel*) or by SDS-PAGE and fluorography to compare the levels of the EGF-R protein (*lower panel*). Numbers under each lane are arbitrary values derived from densitometric scans of the x-ray films.

expressed by the 1483 cells was similar to that expressed by the A431 cells, which serve as the paradigm of EGF-R overexpression, indicating that the 1483 HNSCC cells overexpress the receptor at the protein level, as reported previously (*56*). In contrast, the level of EGF-R protein in the 183 cells was lower by more than 50% than that in the 1483 cells, in agreement with the ligand-binding data and the immunoprecipitation of cell-surface ^{125}I-labeled EGF-R (Fig. 2). Treatment with RA of the three cell lines increased the level of immunoprecipitated EGF-R in each by 14% to 66% (Fig. 3, *lower panel*). Thus, it appears that RA decreased the level of EGF-R mRNA in the 1483 cells without a corresponding decrease in the protein level and increased the level of immunoprecipitable protein in the 183 cells without altering the mRNA level.

Suppression by RA of EGF-R Autophosphorylation in HNSCC Cells

The ability of the EGF-R to undergo autophosphorylation is a measure of the function of the receptor. To determine whether RA treatment of the HNSCC cells altered EGF-R function, the tyrosine protein kinase activity of the EGF-R immunoprecipitated from untreated or RA-treated cells was analyzed by the immune-complex kinase assay. Fig. 3 (*upper panel*) shows that untreated 1483 cells exhibited a fourfold higher kinase activity than did the 183 cells, when normalized to equal cellular protein used for the immunoprecipitation, or 2.2-fold higher when normalized to the amount of EGF-R protein immunoprecipitated from [^{35}S]methionine-labeled cells (Fig. 3, *lower panel*). The EGF-R immunoprecipitated from RA-treated A431, 1483, and 183 cells possessed a lower kinase activity than receptor immunoprecipitated from untreated cells (Fig. 3, *upper panel*). The ability of RA to suppress EGF-R autophosphorylation activity was most prominent in the 183 cells (about 85% inhibition) and less so in the 1483 (about 65% inhibition) and A431 cells (about 50% inhibition). This effect of RA was dose-dependent: 50% inhibition required about 0.1 and 0.28 μM RA in the 183 and 1483 cells, respectively (Fig. 4). The radioactivity associated with the EGF-R in the gels was mostly associated with tyrosine residues, since it was reduced by less than 5% after the gels were immersed in 1 *M* KOH and heated at 80 °C for 1 hour, conditions that remove most phosphate moieties from serine or threonine residues (data not shown).

Effect of RA on the Glycosylation of EGF-R in HNSCC Cells

Like phosphorylation, glycosylation is a posttranslational protein modification that can alter protein function and stability. To determine whether treatment with RA affects the glycosylation of EGF-R, HNSCC cells were metabolically labeled with radioactively labeled monosaccharides and the EGF-R was immunoprecipitated and analyzed by SDS-PAGE and fluorography. Fig. 5 shows that RA treatment of the HNSCC cells decreased the incorporation of [^{3}H]fucose and [^{3}H]glucosamine by about 40% and 60% in the 1483 cells and by 87% and 22% in the 183 cells, respectively. In contrast, the amounts of EGF-R immunoprecipitated from lysates of cells labeled metabolically with [^{35}S]methionine were similar in untreated and treated cells (Fig. 5).

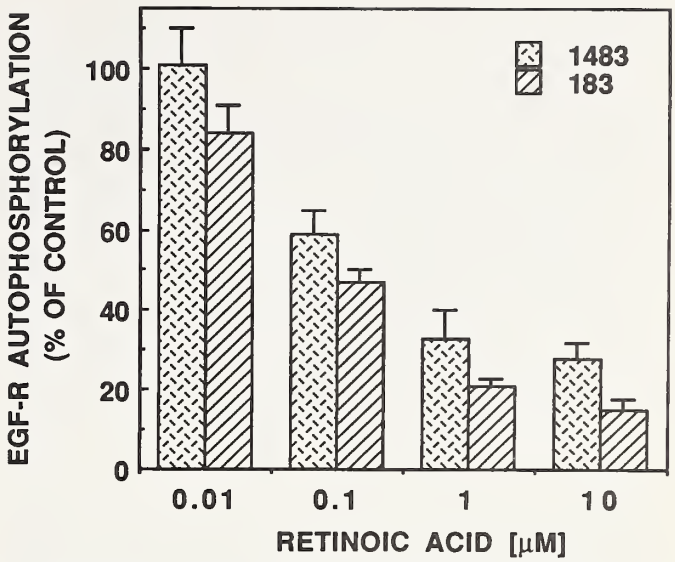

Fig. 4. RA dose-dependent suppression of EGF-R autophosphorylation in human 1483 and 183 HNSCC cells. Cells were grown for 7 days in medium supplemented with 0.01% DMSO or with the indicated RA concentrations. Cells were then harvested and lysed, and their EGF-R was immunoprecipitated and analyzed for autophosphorylating activity. Protein kinase activity in RA-treated samples was estimated from densitometric scans of the autoradiograms and related to that measured in untreated control cells, which was designated as 100%.

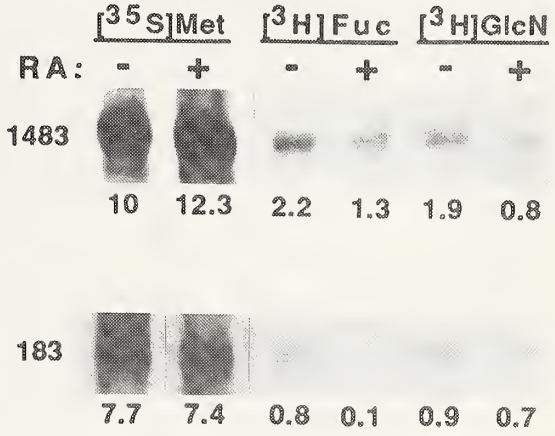

Fig. 5. Suppression by RA of EGF-R glycosylation in human 1483 and 183 HNSCC cells. Cells were grown for 7 days in medium supplemented with 0.01% DMSO (−) or with 1 μM RA (+). Medium was also supplemented with [^{3}H]fucose or [^{3}H]glucosamine (40 μCi/mL) during the last 24 hours of growth, or with [^{35}S]methionine (50 μCi/mL) during the last 12 hours of growth. Cells were then harvested and lysed, and EGF-R was immunoprecipitated as described in Materials and Methods. Immunoprecipitates were analyzed by SDS-PAGE and fluorography. Numbers under each lane are arbitrary values derived from densitometric scans of the x-ray films.

Effect of RA on the Internalization of EGF-R in HNSCC Cells

The extent of internalization of cell surface membrane EGF-R is one mechanism by which EGF-R function can be controlled. Although the 183 cells bound half as much ^{125}I-labeled EGF as the 1483 did (Fig. 2), the rate of internalization of cell-surface-bound ^{125}I-labeled EGF in the 183 cells was higher than that of the 1483 cells, as was the percent of internalized receptor (Fig. 6). Treatment with RA had no significant effect on either the rate or the extent of ^{125}I-labeled EGF-EGF-R complex internalization in the two HNSCC cell lines (Fig. 6). This result is similar to that reported by Jetten (*43*) for 3T6 fibroblasts.

DISCUSSION

Cell growth and differentiation are regulated at various levels. One of the most important sites of regulation is the cell surface membrane, where receptors for exogenous growth factors reside and mediate signal transduction. The EGF-R has been a target for modulation by various exogenous agents in an attempt to either understand its function or interfere with cell growth. Abrogation of EGF-R function was demonstrated with antireceptor antibodies in vitro and in vivo (*63*). Several agents such as ganglioside GM_3 (*64*), erbstatin (*65*), tyrphostins (*66*), and TNF-α (*67*) were found to inhibit the tyrosine protein kinase activity of the receptor. The major finding of this study is the demonstration of the ability of RA to suppress the protein tyrosine kinase activity of the EGF-R in two HNSCC cell lines. This finding is similar to results obtained with human glioblastoma cells (*53,64*) and suggests that this may be one mechanism by which RA can interfere with EGF-R function.

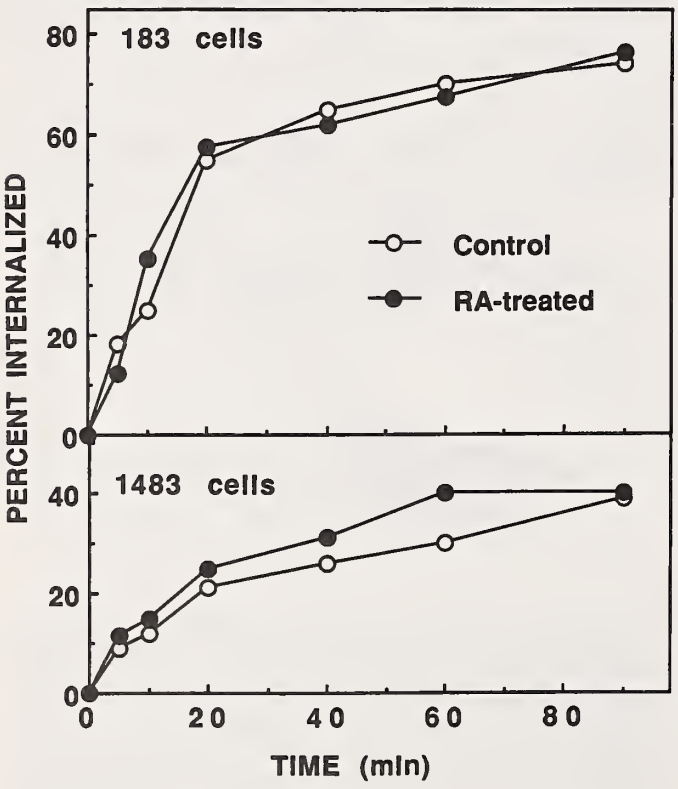

Fig. 6. Internalization of cell-surface-bound ^{125}I-labeled EGF by human 1483 and 183 HNSCC cells. Cells were grown for 7 days in medium supplemented with 0.01% DMSO (control) or with 1 μM RA (RA-treated) and were incubated with ^{125}I-labeled EGF for 45 minutes at 4 °C and then at 37 °C for the indicated times. Amount of internalized ^{125}I-labeled EGF was determined as described in Materials and Methods. Each point represents the average of triplicate assays. SE was less than 5%.

Previous studies have shown that retinoids increased the binding of EGF to the surface of numerous cell types (*34,42,43,46,49*). In some cells this was shown to be the result of a retinoid-enhanced EGF-R biosynthesis (*46,49,50*), a process that might be mediated via nuclear retinoic acid receptors (*51*). An opposite effect, namely a retinoid-induced decrease in EGF binding and in EGF-R synthesis, was observed in a few cell types (*47,48,52*). In other cell lines, such as A431 cells (*42*), cultured human keratinocytes (*68*), and those derived from human lung carcinomas (*69*), RA treatment resulted in no or small changes in EGF binding. In this study, we found that RA decreased somewhat the binding of EGF to the 1483 cells without affecting the binding to 183 cells. Although the EGF-R mRNA level decreased in RA-treated 1483 cells, the amount of EGF-R protein immunoprecipitated from [^{35}S]methionine-labeled cells increased, suggesting that there was no decrease at the protein level. Furthermore, the half-life of the EGF-R protein in the untreated 1483 cells was 16 hours, and in the treated cells it was 17 hours (data not shown), indicating that RA did not alter protein stability. This apparent discrepancy between effects of RA on the EGF-R mRNA and protein may be explained by an increased affinity of the antibody to the less glycosylated EGF-R from RA-treated cells compared to EGF-R from untreated ones or by an increased translatability of the EGF-R mRNA in RA-treated cells.

The effects of EGF on the growth of squamous cell carcinoma (SCC) cells are variable and may depend on the cell type, growth conditions, and EGF-R level on the cell surface. Four SCC cell lines established from tongue tumors did not require EGF for growth (*70*). However, EGF has been shown to inhibit the in vitro growth of A431 cells and some HNSCC cell lines, which overexpress EGF-R (*17,44*). In contrast, EGF-R gene amplification and protein expression have been implicated in tumorigenicity of the A431 epidermoid carcinoma cells in nude mice (*71*). In a previous study using glioblastoma cells, we found that RA suppressed EGF-R protein tyrosine kinase activity in a cell line that was susceptible to the growth inhibitory effect of RA but not in a cell line that was resistant to RA. We concluded that the suppression of kinase activity may have been the cause of growth inhibition (*53*). The present study shows that this may not be the case for the HNSCC cell line 183. Because the autophosphorylation of the EGF-R is essential for signal transduction (*2,7*), the ability

of RA to suppress this process may be responsible in part for the effect of RA on the proliferation of some cells like 1483 HNSCC cells. Arguing against this possibility are the results found with 183 HNSCC cells, which are not inhibited by RA, although the autophosphorylation of EGF-R is inhibited in them more than in the 1483 cells. Thus, it appears that suppression of EGF-R kinase is not sufficient to cause growth inhibition in the 183 cells.

Both the 1483 and the 183 HNSCC cell lines were derived from well-differentiated SCCs (*57*). However, the 1483 cells express a more squamous differentiated phenotype than the 183 cells in that they express a higher level of transglutaminase type I and cholesterol sulfate (*39*) and exhibit a greater envelope competence (Lotan R: unpublished data). Treatment with RA effectively inhibited the squamous differentiation of the 1483 cells (*39,41*) and decreased the level of cholesterol sulfate in the 183 cells (*39*). It is possible, therefore, that the inhibition of EGF-R protein tyrosine kinase is related to the suppression of the squamous differentiation in these HNSCC cells. Several reports studied the relationship between the degree of squamous cell differentiation and the expression of the EGF-R. Thus, in several epidermal and oral cavity SCC cell lines, an inverse relationship was found between ability to bind ^{125}I-labeled EGF and ability to undergo squamous differentiation (formation of cornified envelopes) on addition of Ca^{2+}-ionophore (*72*). Likewise, several clones of A431 cells selected for resistance to the growth-inhibitory effect of EGF were found to express lower levels of EGF-R and to undergo a more extensive squamous cell differentiation than did the parental A431 cells (*73*). These results suggested an inverse relationship between the level of EGF-R and squamous cell differentiation. Further, it was proposed that high protein kinase activity results in a retardation of processes required for squamous differentiation. However, several other studies have reached the opposite conclusion. For example, it was found that among 21 surgical specimens of HNSCC, all those that showed amplification and/or overexpression of EFG-R were histologically well-differentiated SCCs, whereas none of the less differentiated SCCs showed this aberration in EGF-R expression (*13*). Furthermore, in A431 cells EGF increased the production of involucrin, a marker of squamous cell differentiation (*74*), and EGF- or TGF-α-induced EGF-R-dependent phosphorylation of several proteins; it also stimulated squamous cell differentiation, as evidenced by increased cross-linked envelope competence in human lung SCC cells in culture (*75*). In addition, interferon-γ has been shown to induce terminal squamous cell differentiation (increase in the expression of K1 keratin, morphologic changes, and eventual cell death) in A431 epidermoid carcinoma cells and in primary tumoroid cultures from human laryngeal SCCs and to increase the expression of EGF-R mRNA and protein (*76*). These results suggested a correlation between increased EGF-R level and protein kinase activity and enhancement of squamous cell differentiation. If such a correlation exists in the 1483 and 183 HNSCC cells, then it is plausible to suggest that the ability of RA to suppress the protein tyrosine kinase activity in these cells is related to inhibition of squamous cell differentiation.

The mechanism by which RA modulates the phosphorylation of EGF-R in the HNSCC cell lines is not clear. Because the amount of the EGF-R protein is not decreased by RA, it is likely that the effect of RA is posttranslational. This idea is appealing in view of our demonstration that RA decreased the glycosylation of EGF-R in the two HNSCC cell lines. Interestingly, the decrease in the glycosylation of EGF-R in these HNSCC cells is not the result of a general suppression of glycosylation; as we have previously reported, RA enhanced the glycosylation of other cell surface glycoproteins in the 1483 cells and had no effect on the glycoslation of major glycoproteins in the 183 cells (*37*). In A431 cells, the EGF-R core polypeptide (135 kd) was found to be modified cotranslationally by the covalent attachment of 11 *N*-linked oligosaccharide chains to yield a 160 kd precursor. Further processing and addition of peripheral sugars in the Golgi apparatus resulted in the formation of the mature receptor (170 kd) (*3*). Similar results were reported for several oral squamous carcinomas (*77*). The EGF-R isolated from A431 cells was found to contain both high mannose-type and complex-type asparagine-linked oligosaccharide chains. The complex chains are predominantly of the tri- and tetra-antennary species and contain low amounts of sialic acid, high amounts of fucose, and α-linked *N*-acetylgalactosamine residues, the blood group A determinant (*78*). The relationship between glycosylation and function of the EGF-R has been studied in a number of SCC cell lines. It was reported that EGF-binding activity requires *N*-glycosylation and occurs in the Golgi apparatus at an early stage preceding the processing of high-mannose chains by mannosidase II (*77*). In contrast, the acquisition of the tyrosine kinase autophosphorylation activity of EGF-R does not require *N*-glycosylation (*79*). However, a possible involvement of *N*-acetylgalactosamine residues on the EGF-R in determining affinity for EGF and protein tyrosine kinase activity has been proposed (*80*). We speculate that the modulation of EGF-R glycosylation may alter its structure, affecting the phosphorylation of the receptor and the signal transduction pathway that it mediates. Increasing evidence suggests that tyrosine kinase growth factor receptors play multiple roles in growth regulation and may be important in both mitogenesis and differentiation. The altered glycosylation of EGF-R may be important in the role of the receptor in squamous cell differentiation.

Recently, it has been shown that tyrosine kinase inhibitors (tyrphostins) exerted antiproliferative effects on the growth of human HNSCC cells in vitro and in nude mice (*66*). Additional studies with a larger number of HNSCC cell lines responsive and unresponsive to RA-induced growth inhibition and squamous differentiation modulation are required to determine whether the suppression of EGF-R protein kinase activity by RA is causally related to the effects of RA on cell growth, differentiation, or both.

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13-*cis*-Retinoic Acid and Cancer Chemoprevention

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ABSTRACT—Chemoprevention is the newest strategy for controlling and managing cancer. At present, the multistep character of epithelial carcinogenesis makes this disease process the most amenable to chemopreventive interventions, which occur in the postinitiation, preinvasive phases. Chemoprevention study has focused on oral carcinogenesis because of its excellent preclinical models, well-defined premalignant phase (leukoplakia), ease of monitoring, and link through field carcinogenesis to other epithelial carcinogeneses of the upper and lower aerodigestive tract. Retinoids, the derivatives of vitamin A, are the most-studied chemopreventive agents, and 13-*cis*-retinoic acid is the best-studied chemopreventive retinoid. Laboratory study of the newly discovered nuclear receptors of retinoic acid is closing in on the precise mechanism of retinoid action. Only 13-*cis*-retinoic acid, at high doses, has established chemopreventive activity, which is in suppressing oral premalignancy and preventing second primary head-and-neck tumors. Preclinical and clinical work in the other aerodigestive sites of the lung and esophagus are at an early phase of study with no conclusive results currently available. High-dose 13-*cis*-retinoic acid also has achieved significant activity in preventing invasive carcinomas of the skin. High-dose 13-*cis*-retinoic acid, however, is not ideal for widespread chemoprevention approaches because of its toxicity. The toxicity-to-risk balance is delicate and complicated. In extremely high-risk patients, high-dose 13-*cis*-retinoic acid ultimately may play a clinical role. Unfortunately, study of less-toxic agents, such as β-carotene, has yet to produce a record of established activity. Future directions of chemoprevention study will investigate less-toxic retinoids, natural nontoxic agents, lower and better tolerated doses of 13-*cis*-retinoic acid, and combinations of these agents. Chemoprevention is still in its infancy, but its first steps in humans were taken by 13-*cis*-retinoic in preventing invasive carcinoma. [J Natl Cancer Inst Monogr 13:111–115, 1992]

After decades of rigorous investigation of countless cancer chemotherapeutic regimens, many cancers, including those of the lung and head and neck, remain beyond our clinical ability to control them. Despite therapeutic advances and intensive efforts in tobacco-cessation education and counseling, overall survival rates for patients with aerodigestive tract cancers have improved only marginally over the past thirty years (*1*).

The 5-year survival rate for patients with advanced lung cancer is less than 5% (*1*). Of all patients initially "cured" of early stage head-and-neck cancer, 3% to 5% per year will develop fatal second primary tumors (*2*). Standard local therapy of oral premalignancy is virtually meaningless in high-risk cases because of this disorder's characteristic appearance in multiple foci, some of which can appear normal to standard clinical observation but can have microscopic signs of carcinogenesis. Researchers, therefore, have begun to focus serious attention on the new field of chemoprevention for controlling some of these intractable and deadly diseases.

The early focus of chemoprevention study itself has been on preventing cancers of the upper aerodigestive tract. For several reasons the upper aerodigestive tract lends itself to this work. First, excellent preclinical systems exist for studying carcinogenesis in this region. Second, the relationship between upper aerodigestive tract premalignancy and resulting invasive cancers is well established (*3,4*). Third, the upper aerodigestive tract is relatively easily and noninvasively monitored. Last, and most important clinically, carcinogenesis in the upper aerodigestive tract is closely related to carcinogenesis in the rest of the aerodigestive tract, i.e., the esophagus and lungs. This relationship is based on common etiology—tobacco carcinogens (*2*).

The foundation for all chemoprevention approaches involves two breakthrough concepts in the biology of carcinogenesis. One seminal concept is that of "field" carcinogenesis (*5*). The postulate here is that carcinogenic exposure can occur and initiate carcinogenesis throughout a wide field. This fits the pattern of upper aerodigestive tract cancer: tobacco usage can initiate carcinogenesis in a field that extends from the oral cavity (part of the upper aerodigestive tract) down to and into the lungs. The other seminal concept is that of multistep carcinogenesis. This postulate is that carcinogenesis evolves through several subtle sequential phases, beginning with initiation in a precancerous phase and advancing through premalignant steps of promotion, before progressing to the end-stage of invasive cancer. This pattern also fits the upper aerodigestive tract. Oral premalignancy has an established relationship with the multiphasic development of invasive cancer of the head and neck (*2–4,6*).

Basic postulates of the chemoprevention approach are that 1) intervention with a chemopreventive agent will help

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prevent the fieldwide appearance of invasive cancer and that 2) carcinogenesis can be arrested or reversed in precancerous stages (*2,7*). So far, only one tested agent has provided positive clinical chemoprevention data in support of both of these essential postulates—the synthetic retinoid 13-*cis*-retinoic acid.

PRECLINICAL STUDY OF RETINOIDS IN THE UPPER AERODIGESTIVE TRACT

Although not specific to the upper aerodigestive tract, some extraordinary recent laboratory research has identified the nuclear retinoid acid receptors. Investigation of the processes of these receptors well may reveal the fundamental mechanism of retinoid action (*8*). The retinoic acid receptors, which have been identified as members of the well-studied family of steroid receptors, are gene products that bind either with endogenous low levels of retinoic acid or with pharmacologic levels of retinoids. Then, after undergoing conformational changes, the retinoic-acid-loaded retinoic acid receptors re-enter the DNA of specific chromosomes and, through transactivation, exert their effects on gene transcription activity. Here we stand at the threshold of well-documented effects of vitamin A and other retinoids both on normal tissue development and on carcinogenic tissue changes. Retinoic acid receptor-mediated gene activity may help make an eye strong or arrest the progression of carcinogenesis in a patient with dysplastic premalignant oral lesions (*9*).

Our most useful preclinical models for testing agents specifically against upper aerodigestive tract carcinogenesis are those of the Syrian golden hamster's buccal pouch and tongue (*6,10,11*). Positive preclinical results have been achieved by β-carotene, selenium, vitamin E, and vitamin A (retinol), and various other retinoids (*10–15*). Only the retinoids have achieved activity in both models.

Lung carcinogenesis studies conducted over 60 years ago by Wolbach and Howe (*16*) and recently by Mehta et al (*17*) have demonstrated retinoid activity in the lung. Retinoid results in esophageal animal models, however, are not clear. Because the upper two-thirds of the esophagus shares epithelium with the upper aerodigestive tract and because carcinogenesis in the two areas shares features of biology and etiology, one might expect that agents active against upper aerodigestive tract carcinogenesis would be active in esophageal cancer systems as well. Some in vitro and animal esophageal data indicate retinoid activity. But other animal data are negative, and some even indicate that retinoids can promote esophageal carcinogenesis (*18,19*).

Volumes of general positive preclinical data on retinoid anticancer activity have been reviewed in detail elsewhere (*20–23*). These data plus the positive preclinical results referred to above led to several clinical trials of 13-*cis*-retinoic acid for chemoprevention in the upper aerodigestive tract and a few in the lungs. Conflicting data from esophageal preclinical systems, in addition to the difficulty in monitoring premalignancy in this region, so far have limited testing of retinoids in the esophagus to one large-scale phase III trial of a retinol-combination by Munoz et al. in China (*24*).

A BRIEF CLINICAL HISTORY OF 13-*CIS*-RETINOIC ACID IN THE UPPER AERODIGESTIVE TRACT

Most clinical trials of chemopreventive agents in the upper aerodigestive tract are fairly recent, and the two best-studied single agents are 13-*cis*-retinoic acid and beta-carotene (*6,23,25–40*). Although limited nonrandomized trials of beta-carotene indicate this natural agent's potential, its chemopreventive effectiveness is unconfirmed (*35, 36, 38–40*). Only 13-*cis*-retinoic acid and retinol have been established statistically to have significant chemopreventive activity in the upper aerodigestive tract (*32,37*).

Favorable data on four retinoids in oral premalignancy have been reported for over 20 years (*25–34,36*). Only three randomized trials of retinoids have been reported, however: two using 13-*cis*-retinoic acid (*32,33*) and one using high-dose natural vitamin A (*37*).

Our group reported the first randomized trial of 13-*cis*-retinoic acid (administered in high doses) versus placebo in a high-risk subject population with a predominance of dysplastic oral leukoplakia (*32*). Our results of highly significant 13-*cis*-retinoic acid activity were so dramatic, we stopped this study prematurely after accrual of only 44 of 60 planned evaluable patients. Of our retinoid group, two-thirds responded clinically and half responded with histologically documented reversal of dysplasia. Frequently intolerable toxicity at highest doses and a 50% relapse rate after discontinuing the drug led us to design a second trial in subjects with high-risk oral premalignancy to study less toxic or nontoxic maintenance.

Our second trial began with a short-term induction phase of high-dose 13-*cis*-retinoic acid (1.5 mg per kg per day), followed by randomization of responding subjects to either the natural nontoxic agent β-carotene (30 mg per day) or to low-dose 13-*cis*-retinoic acid (0.5 mg per kg per day) as maintenance for 9 months (*33*). Our induction results paralleled the established 67% response rate to retinoid. Data from our maintenance phase indicated that low-dose 13-*cis*-retinoic acid was significantly more active than was β-carotene in preventing lesion progression or relapse and in producing further responses. Low-dose 13-*cis*-retinoic acid was tolerated well: 80% of 13-*cis*-retinoic acid maintenance subjects completed the 9-month regimen without dose reductions, and no patient in either arm dropped out of the study because of toxicity.

Stich et al. (*37*) recently reported the one randomized trial, a small one, of high-dose (200 000 IU per week) vitamin A. It was conducted in a high-risk group of Asian betel nut chewers with oral premalignancy. Stich's trial achieved a remarkable 57% clinical complete response rate without clinical toxicity.

Laryngeal papillomatosis is the only other upper aerodigestive tract lesion that has undergone chemo-

prevention study (*23,41,42*). Work has been limited by a variety of factors, including more invasive monitoring requirements, etiologic differences from other upper aerodigestive tract preneoplasias, low incidence, and low transformation rates. The synthetics 13-*cis*-retinoic acid and etretinate have been assessed in two trials involving a total of 48 patients with this hyperproliferative lesion. The overall clinical objective response rate (papilloma reversal) to each agent alone was 67% (*41,42*). Despite positive data in extant disease, adjuvant 13-*cis*-retinoic acid did not prevent lesion recurrence in a recent small trial (*43*).

The earliest preclinical retinoid work in cancer conducted by Wolbach and Howe in 1925 (*16*) supported retinoid use in suppressing lung cancer carcinogenesis. These and other primary epidemiologic and preclinical studies led to several lung cancer chemoprevention trials. Investigators from France reported a series of nonrandomized trials using etretinate in chronic smokers with significant bronchial metaplasia as determined by multisite bronchoscopic biopsy (*44–46*). Significant regression of subjects' metaplastic lesions was found on repeat bronchoscopy after 6 months of therapy ($P < .01$). The reversal of metaplasia was more marked and more rapid in the 5% to 10% of patients who stopped smoking while receiving etretinate therapy, suggesting for the first time a beneficial interaction of smoking cessation and retinoid chemoprevention. We are currently conducting a rigorous, placebo-controlled trial of 13-*cis*-retinoic acid in chronic smokers with bronchial metaplasia (*47*). In contrast to four reported positive trials evaluating histologic endpoints of metaplasia, two synthetic retinoid (13-*cis*-retinoic acid, etretinate) trials evaluating sputum cytologic atypia as the intermediate and only endpoint were negative (*23,48*).

We also have conducted a randomized, placebo-controlled trial of high-dose 13-*cis*-retinoic acid as adjuvant therapy in patients "cured" of primary head and neck squamous cell carcinoma. These patients have high rates of relapse, distant metastasis, and, most dangerous of all, second primary tumor development. Earlier data from this trial have been reported in detail elsewhere (*49*), but here we will update our results after nearly 4 years of median follow-up.

As previously reported, our most significant result was in the prevention of second primary tumors. Despite only 1 year of treatment and a high drop-out rate of 50% in the treatment group, the significant reduction in the rate of second primary tumor development in the 13-*cis*-retinoic acid group continues (28% versus 6%, $P<.005$). No treated patients developed multisite second primary tumors, and over 75% of all second primary tumors occurred in the aerodigestive tract. This site-dispersal pattern is consistent with that reported in other studies and with the concept of field carcinogenesis initiated and promoted by tobacco carcinogens (*2*).

In our first published report of this trial, we stated that 13-*cis*-retinoic acid did not produce a significant reduction in overall failure rate or in local, regional, and distant relapses plus second primary tumors (*49*). Now, after nearly 4 years of follow-up the overall failure rate is significantly lower in the 13-*cis*-retinoic acid group.

A recent randomized placebo-controlled adjuvant trial of retinyl palmitate achieved a significant reduction in disease recurrence in patients "cured" of early stage lung cancer (*50*).

Toxicity is the greatest limiting factor in the use of 13-*cis*-retinoic acid for chemoprevention (*23*). This concern is not based primarily on retinoids' well-known teratogenicity, however, because the vast majority of subjects at high risk of aerodigestive tract cancers are women beyond child-bearing potential and men. The largest toxic concern is that of mucocutaneous side effects. Chemoprevention trials involve relatively healthy individuals, and many will not tolerate drug-related discomfort (*51*). For this reason, in our 13-*cis*-retinoic acid investigation we have tried reducing doses to the lowest effective levels to reduce mucocutaneous toxicity to the greatest extent possible. Additional efforts include adding toxicity-mitigating agents, such as alpha-tocopherol, to effective 13-*cis*-retinoic acid dose levels (*52*). These approaches would help highest risk subjects who are more apt to tolerate toxicity and would open the door for 13-*cis*-retinoic acid chemoprevention in subjects at lower risk.

Concerns for toxicity also encourage us and other investigators to continue searching for natural, nontoxic alternatives to 13-*cis*-retinoic acid for chemoprevention in the upper aerodigestive tract and lung. Beta-carotene has produced interesting results but remains questionable as a single agent. We are planning a randomized, long-term trial of the completely nontoxic combination of retinol plus β-carotene for reversing oral premalignancy and preventing invasive cancer. Unlike our two earlier randomized oral leukoplakia trials, this trial disposes entirely of toxic high doses of 13-*cis*-retinoic acid. We have included as a positive control a low-dose 13-*cis*-retinoic acid group (reduced from our previous maintenance trial dose) because of this agent's established activity levels and our clinical belief that it would be unethical to administer placebos to the high-risk subjects proposed for this trial.

CONCLUSION

Data from the several randomized trials of 13-*cis*-retinoic acid in the upper aerodigestive tract establish 13-*cis*-retinoic acid as an effective chemopreventive agent against tobacco-related carcinogenesis in this region. In another landmark chemoprevention trial, Kraemer et al. (*53*) used high-dose 13-*cis*-retinoic acid to achieve a significant reduction in new tumors in xeroderma pigmentosum patients "cured" of skin cancer. Chemoprevention with 13-*cis*-retinoic acid may be appropriate for subjects with high-risk dysplastic oral lesions and for patients "cured" of head and neck cancer and at high risk of developing second primary tumors and for highest risk patients "cured" of skin cancer.

Worldwide tobacco use continues at staggering levels—one billion people smoke and 600 million people chew

tobacco products. The carcinogenic consequences are equally staggering: in the United States alone, fully one-third of the over 500 000 cancer deaths each year are caused by tobacco-related aerodigestive tract cancers. The 13-*cis*-retinoic acid benefits mentioned above could result potentially in saving tens of thousands of lives annually in the United States and millions of lives throughout the world.

Having a standard of effectiveness allows investigators to employ 13-*cis*-retinoic acid in the study of biomarkers of potential intermediate endpoints of carcinogenesis. Established response to 13-*cis*-retinoic acid provides the necessary correlative clinical data to complement any molecular-cellular changes indicated by potential intermediate endpoint biomarkers. This research is at the forefront of current chemoprevention study because of the need to make large-scale, phase III testing of new chemopreventive regimens more efficient. Currently these trials involve tremendous expense, time, and numbers of subjects because their only valid endpoints are invasive cancer. Valid intermediate endpoints, reflected by biomarkers, may one day replace the long-term endpoint of malignancy and thereby allow us to reduce significantly the overall expense of phase III trials. In the national atmosphere of growing budgetary constraints, advances in chemoprevention may depend on streamlining phase III trials through biomarkers (*54*).

The consistently active agent 13-*cis*-retinoic acid may become standard chemoprevention for a wide variety of tobacco-related cancers, or it may only be a pioneer paving the way toward better, safer chemopreventive approaches. Whichever the case, certainly 13-*cis*-retinoic acid has shown that well-designed randomized chemoprevention trials are feasible and promising avenues for increasing our ability to manage and control neoplasia.

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Epidermal Growth Factor Receptor Monoclonal Antibodies Inhibit the Growth of Lung Cancer Cell Lines[1]

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ABSTRACT—The ability of monoclonal antibody (MAb 108), an immunoglobulin G (IgG)2a against the epidermal growth factor receptor (EGF-R), to interact with lung cancer cell lines was investigated. ^{125}I-EGF bound with high affinity to non-small-cell lung cancer (NSCLC) cells, and MAb 108 inhibited specific binding of nine NSCLC cell lines in a dose-dependent manner (IC_{50} = 0.3–3 μg per ml). ^{125}I-MAb 108 bound with high affinity (kd = 2 nM) to a single class of sites (Bmax = 70 000 per cell) using NSCLC neuroendocrine cell line NCI-H460. Specific ^{125}I-MAb 108 binding was inhibited with high affinity by MAb 108 but not by a control antibody IgG using large-cell carcinoma cell line NCI-H1299. ^{125}I-MAb 108 binding was not internalized at 37 °C using NSCLC neuroendocrine cell line NCI-H460 and adenocarcinoma cell line NCI-H23. Also, 1 μg per ml of MAb 108 but not of a control IgG inhibited the clonal growth of NCI-H23 and squamous cell carcinoma cell line NCI-H157 in vitro. Also, MAb 108 inhibited xenograft formation of cell lines NCI-H460, NCI-H157, and NCI-H727 in nude mice in vivo. After a palpable tumor had formed using NCI-H460 cells, injection of 100 μg of MAb 108 (intraperitoneally three times weekly) inhibited xenograft volume in nude mice by approximately 50%. These data suggest that MAb 108 may interact with EGF receptors on lung cancer cell lines and inhibit NSCLC proliferation. [J Natl Cancer Inst Mongr 13:117–123, 1992]

The epidermal growth factor receptor (EGF-R) is a 1186 amino acid protein comprised of a 621 amino acid extracellular domain that contains the epidermal growth factor (EGF) binding site, followed by a 23 amino acid transmembrane domain and a 542 amino acid cytosolic domain that has tyrosine kinase activity (*1*). When EGF binds to the EGF-R, tyrosine residues are phosphorylated on substrates such as the C-terminal of the EGF-R and phosphatidyl inositol is turned over, cytosolic Ca^{2+} is elevated, the cytoplasmic pH is increased, the EGF-R is internalized, and target cells become committed to synthesize DNA and divide (*2–5*). The EGF-R has structural similarities to the v-erbB oncogene (*6*).

The EGF-R has been detected in many non-small-cell lung cancer (NSCLC) but not in small-cell lung cancer (SCLC) cells (*7,8*). NSCLC cells synthesize transforming growth factor α (TGFα), which binds to the EGF-R (*9*). Because monoclonal antibodies (MAbs) against TGFα inhibit the growth of some NSCLC cells, TGFα may be an autocrine growth factor for some NSCLC cells (*10*).

It may also be possible to inhibit the growth of EGF-R positive cells using MAbs against the EGF-R. Previously, MAbs such as 528 and 225 inhibited the growth of A431 vulvar squamous carcinoma (A431) and colon adenocarcinoma (SW948) xenografts in athymic mice (*11,12*). In particular, MAb 225 inhibited EGF-R binding and was internalized, resulting in serine and threonine, but not tyrosine phosphorylation, of the EGF-R (*13,14*). Also, MAb 108 inhibited the growth of oral epidermoid (KB) carcinoma xenografts in nude mice (*15*). MAb 108 inhibited high-affinity EGF-R binding, EGF-R tyrosine phosphorylation, elevation of cytoplasmic pH, and elevation of cytosolic Ca^{2+} (*16*). Here the ability of MAb 108 to inhibit EGF-R binding and NSCLC growth was investigated.

MATERIALS AND METHODS

Cell Culture

Human lung cancer cell lines were cultured in serum-supplemented medium (RPMI-1640) containing 10% heat-inactivated fetal bovine serum at 37 °C as described previously (*17*). The NSCLC cell lines were adherent and were split weekly in a 1:20 ratio using trypsin/EDTA (*17*). Routinely, the cells had greater than 90% viability and were mycoplasma free. The cells were used when they were in an exponential growth phase. Ten lung cancer cell lines that bound EGF with high affinity were cultured.

Receptor Binding

The ability of MAb 108 to inhibit EGF-R binding was investigated as described previously (*18*). MAb 108

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(IgG2a) was produced after injection of mice with CHO cells transfected with a plasmid bearing a cDNA construct of the human EGF-R (10^6 receptors per cell). Spleen cells of immunized mice were fused with NSO-1 myeloma cells. The hybridomas secreting MAbs against the EGF-R were selected, cloned, and injected into mice (*16*). Ascites fluid was ammonium sulfate precipitated and MAb 108 purified on a protein A-Sepharose column (Sigma Chemical Co.; St. Louis, Mo.). The NSCLC cells were harvested after treatment with trypsin/EDTA and 5×10^4 cells were added to 24-well plates, which were pretreated with 10 μg of human fibronectin (Sigma Chemical Co.). After a monolayer of cells had formed (3 days), the cell number was determined and receptor binding studies were conducted. The cells were washed four times with SIT media (RMPI-1640 containing 3×10^{-8} M Na_2SeO_3, 5 μg per mL insulin, 10 μg per mL transferrin, Sigma Chemical Co.) and incubated with ^{125}I-EGF in receptor binding buffer (SIT containing 0.1% bovine serum albumin; Calbiochem, LaJolla, Calif.). EGF (Toyoba, New York, N.Y.) and MAb 108 were iodinated using the chloramine T procedure and ^{125}I-EGF (20 μCi per μg) and ^{125}I-MAb 108 (20 μCi per μg) purified using gel filtration techniques. After 30 minutes at 37 °C, free radiolabeled protein was removed and the cells that contained bound growth factor dissolved in 0.2 *N* NaOH and counted in a gamma counter. Control MAb IgG (IgG2a) was purchased from Sigma Chemical Co.

Clonagenic Assay

Cell lines NCI-H23, NCI-H157, NCI-H727, NCI-H838, and NCI-H1299 were harvested and tested in the agarose cloning system described previously (*19*). The base layer consisted of 3 mL of 0.5% agarose in SIT medium containing 5% fetal bovine serum in six-well plates (Falcon, Oxnard, Calif.). The top layer consisted of 3 mL of SIT medium in 0.3% agarose, the peptide(s) doubly concentrated and 1×10^4 single viable cells. For each cell line and peptide concentration, triplicate wells were plated. After 2 weeks, 1 mL of 0.1% *P*-iodonitrotetrazolium violet was added, and after 16 hours at 37 °C the plates were screened for colony formation; the number of colonies larger than 100 μm in diameter were counted using an Omnicon image analysis system (Bausch and Lomb, Rochester, N.Y.).

Growth Studies in Vivo

The ability of MAb 108 to inhibit xenograft formation in nude mice was investigated using the techniques described previously (*20*). Female athymic Balb/c nude mice, 4 to 5 weeks old, were housed in a pathogen-free, temperature-controlled isolation room, and the diet consisted of autoclaved rodent chow and autoclaved water given ad libitum. NCI-H157, NCI-H460, or NCI-H727 cells (1×10^7) were injected into the right flank of each mouse by subcutaneous injection (*21*). Palpable tumors were observed in approximately 90% of the mice after 14 to 28 days. Phosphate buffered saline (PBS) (100 μL), MAb 108 in PBS, or the IgG control in PBS was injected intraperitoneally. The tumor volume (height × width × depth) was determined weekly by calipers and recorded. When the tumor became necrotic, the growth studies were terminated.

When the growth studies were terminated, some mice were injected with ^{125}I-MAb 108 or ^{125}I-IgG (10^6 cpm). After 1 week, the tumor and several organs were dissected, weighed, and counted. The counts per minute of ^{125}I-MAb 108 or ^{125}I-IgG bound per mg wet tissue weight was calculated.

RESULTS

MAb 108 Binding Studies

Previously, we found that ^{125}I-EGF bound with high affinity to six NSCLC cell lines and to a lung carcinoid (*18*). Here specific ^{125}I-EGF binding was inhibited in a dose-dependent manner by EGF and MAb 108 but not by a control antibody IgG using lung carcinoid NCI-H727. Fig. 1 shows that specific ^{125}I-EGF binding was minimally inhibited by 1 ng per mL EGF but was maximally inhibited by 1 μg per mL EGF. The IC_{50} for EGF was 30 ng per mL. Also MAb 108, but not control antibody IgG, inhibited specific ^{125}I-EGF binding in a dose-dependent manner. The IC_{50} for MAb 108 to inhibit specific ^{125}I-EGF

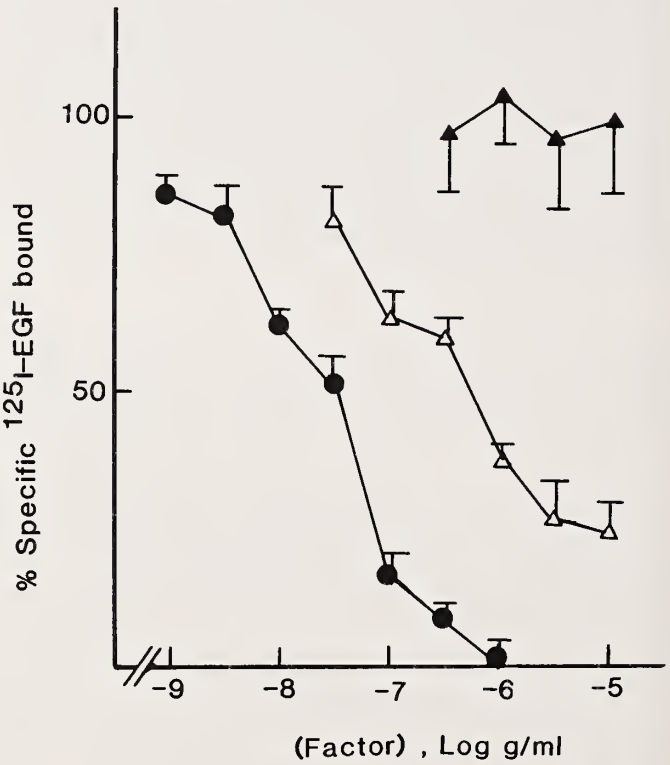

Fig. 1. Inhibition of ^{125}I-EGF binding. The ability of EGF (●), MAb 108 (△), and the IgG control (▲) to inhibit specific ^{125}I-EGF binding to lung carcinoid cell line NCI-H727 was determined. The mean value ± standard error of the mean (SE) of four determinations is indicated.

binding was 0.5 μg per mL, but even high doses such as 10 μg per mL did not totally inhibit radiolabeled EGF binding. These data indicate that ^{125}I-EGF binding is inhibited with high and moderate affinity, respectively, by EGF and MAb 108.

^{125}I-MAb 108 bound with high affinity to lung carcinoid cell line NCI-H727. Fig. 2 shows that ^{125}I-MAb 108 binding was inhibited in a dose-dependent manner by MAb 108 and EGF. Little specific binding was inhibited by 1 ng per mL MAb 108 or EGF whereas specific ^{125}I-MAb binding was totally inhibited by 1 μg per mL MAb 108; 1 μg per mL EGF inhibited most of the ^{125}I-MAb 108 binding. The IC_{50} values for MAb 108 and EGF were 30 and 400 ng per mL, respectively. ^{125}I-MAb 108 binding was not inhibited by control antibody IgG. Therefore ^{125}I-MAb 108 binding is inhibited with high and moderate affinity by MAb 108 and EGF, respectively.

Similar data were obtained for NSCLC cell lines (Table 1). MAb 108 inhibited specific ^{125}I-EGF binding to adenocarcinoma (ADLC-5M2, NCI-H838, NCI-H23, and NCI-H1264), large cell carcinoma (NCI-H1299), and squamous cell carcinoma (NCI-H157, EPLC-32M1, and EPLC-65H) with IC_{50} values ranging from 300 to 3000 ng per mL. In contrast, MAb 108 was more potent at inhibiting specific ^{125}I-MAb 108 binding with IC_{50} values ranging from 40 to 400 ng per mL. These data indicate that for all cell lines examined, MAb 108 was more potent at inhibiting ^{125}I-MAb 108 than ^{125}I-EGF binding.

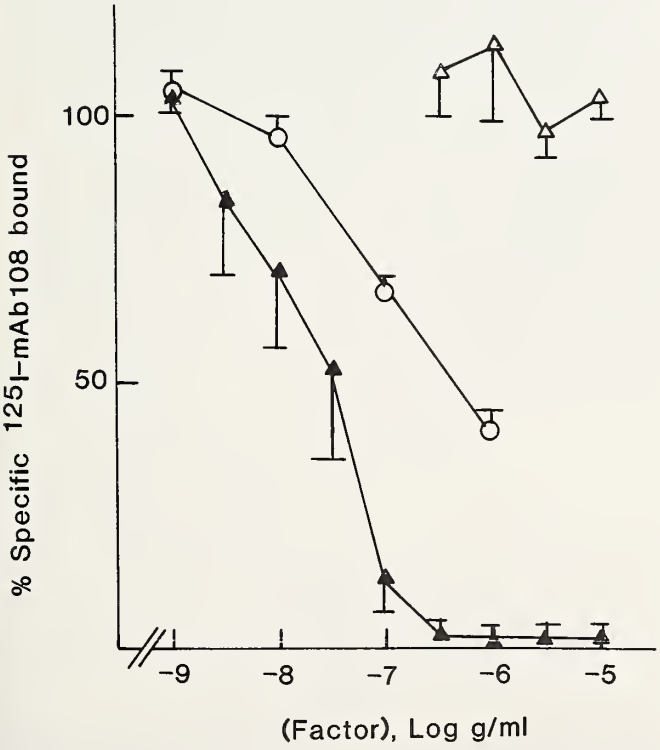

Fig. 2. Inhibition of ^{125}I-MAb 108 binding. The ability of EGF (○), MAb 108 (▲), and the IgG control (△) to inhibit specific ^{125}I-MAb 108 binding to NCI-H727 was determined. The mean value ± SE of four determinations is indicated.

Table 1. Binding to NSCLC cells*

Cell line	^{125}I-EGF(IC_{50})	^{125}I-MAb 108(IC_{50})
Adenocarcinoma		
NCI-H23	n.d.†	90
NCI-H838	1000	400
NCI-H1264	2000	n.d.
ADLC-5M2	500	n.d.
Large cell carcinoma		
NCI-H1299	350	40
Squamous cell carcinoma		
EPLC-32M1	1000	n.d.
EPLC-65H	2000	200
NCI-H157	3000	60
NSCLC neuroendocrine		
NCI-H460	300	200
Lung carcinoid		
NCI-H727	1000	70

*Cells were plated in 24-well plates and the ability of MAb 108 to inhibit specific ^{125}I-EGF and ^{125}I-MAb 108 binding determined. The mean value IC_{50} (ng/mL) of three determinations is indicated.

†n.d. = not determined.

The binding of ^{125}I-MAb 108 appeared saturable (Fig. 3, left). A Scatchard plot of the specific binding data (Fig. 3, right) was linear and indicated that ^{125}I-MAb 108 bound with high affinity (kd = 1.2 nM) to a single class of sites (Bmax = 70 000 per cell) using cell line NSCLC neuroendocrine cell line NCI-H460. Previously, we found that ^{125}I-EGF bound with high affinity to 110 000 sites per cell using cell line NCI-H727 (*18*).

^{125}I-EGF was rapidly internalized at 37 °C after binding to cell line NCI-H727 (*18*). Fig. 4 shows that ^{125}I-MAb 108 slowly bound to cell lines NCI-H23 and NCI-H460 at 4 °C. After 2 hours, approximately 86% and 90% of the radiolabeled MAb 108 could be dissociated from the NCI-H23 and NCI-H460, respectively, by dilute acid/sodium chloride and was membrane bound. In contrast, 14% and 10% of the ^{125}I-MAb was not acid dissociable using NCI-H23 and NCI-H460, respectively, and was solubilized when the cell was dissolved in dilute base. Therefore at 4 °C most of the bound ^{125}I-MAb was not internalized and bound to the cell surface. Similarly, if NCI-H23 and NCI-H460 were first incubated with ^{125}I-MAb 108 at 4 °C for 2 hours, then incubated at 37 °C for 5 minutes, only 24% and 15% of the ^{125}I-MAb 108 was internalized using NCI-H23 and NCI-H460, respectively. These data indicate that at either 4 °C or 37 °C little MAb 108 was internalized.

MAb 108 and Lung Cancer Growth

The effects of MAb 108 on NSCLC growth was investigated in vitro. Table 2 shows that, using a clonogenic assay, colonies formed using cell lines NCI-H23 and NCI-H157. EGF (100 ng per mL) slightly increased NCI-H23 growth and significantly increased NCI-H157 growth. MAb 108 slightly decreased the number of colonies at a 0.1 μg per mL concentration and significantly decreased the number of colonies formed at 1 μg per mL. At 1 μg per mL, MAb 108 significantly decreased NCI-H23 and NCI-

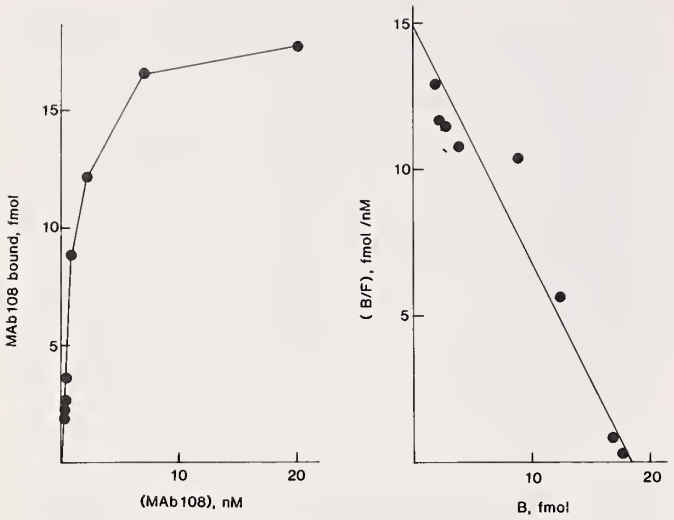

Fig. 3. Binding of ^{125}I-MAb 108. Left: The amount of ^{125}I-MAb 108 bound to NCI-H460 (1.4×10^5 cells) specifically was determined as a function of MAb 108 concentration. Right: Scatchard plot of the specific binding data.

H157 to 50% and 48%, respectively, relative to control values. In contrast, control IgG slightly increased the colony number at a 1 μg per mL concentration. These data indicate that EGF and control antibody IgG stimulate colony formation whereas MAb 108 inhibits growth. Similar data were obtained using cell lines NCI-H727, NCI-H838, and NCI-H1299.

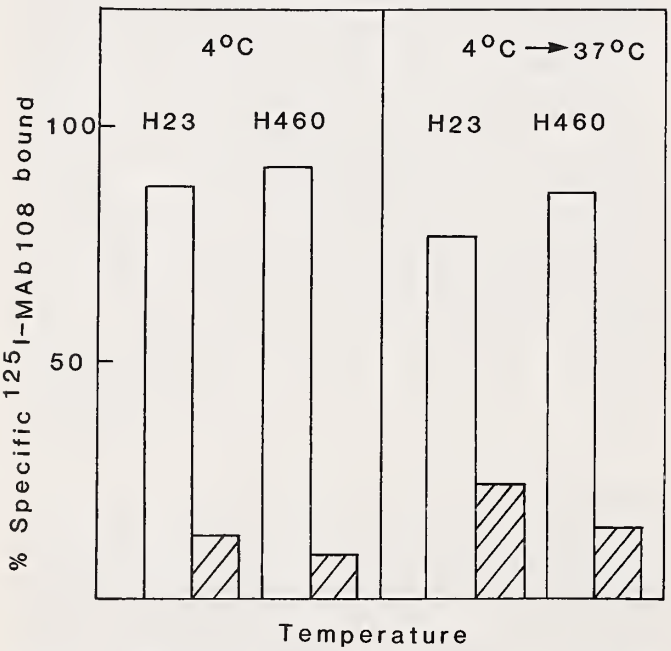

Fig. 4. Internalization of ^{125}I-MAb 108. ^{125}I-MAb 108 was incubated with cell line NCI-H23 and NCI-H460 for 2 hours at 4 °C or 2 hours at 4 °C plus 5 minutes at 37 °C. The percentage of specific ^{125}I-MAb 108 bound to the cell surface (□) and internalized (▨) was determined as described previously (*18*).

Table 2. Effect of agents on NSCLC growth in vitro*

Agent	Colonies using cell line† NCI-H23	NCI-H157
None	20 ± 5(100%)	66 ± 4(100%)
EGF, 100 ng/mL	23 ± 4(115%)	156 ± 24(236%)‡
MAb 108, 0.1 μg/mL	20 ± 1(100%)	49 ± 6(74%)
MAb 108, 1μg/mL	10 ± 2(50%)‡	32 ± 1(48%)‡
IgG, 1μg/mL	25 ± 6(125%)	95 ± 12(144%)

*The mean colony count ± standard error of the mean (SE) of three determinations for cell line NCI-H23 and NCI-H157 is indicated.

†The agent added is indicated on the left.

‡$P < .05$.

Also, the effects of MAb 108 on NSCLC growth were investigated in vivo. Fig. 5 shows that tumors (8 mm^3) formed 3 weeks after implantation of NCI-H727 cells into nude mice. The tumors grew exponentially, and after 9 weeks the mean tumor volume in the control was approximately 1000 mm^3. Similar results were obtained when low concentrations of MAb 108 (10 μg, intraperitoneal) were administered 1 day after tumor cell implantation. When moderate (100 μg) or high (1 mg) quantities of MAb 108 were injected intraperitoneally on day 2, the tumor volume was reduced by approximately 60% at weeks 4 through 9. These data indicate that the effects of MAb 108 in vivo are dose dependent.

The route of administration of MAb 108 was investigated. Fig. 6 shows that in cell line NCI-H460, xenografts formed after 2 weeks (8 mm^3) and grew so that at week 6

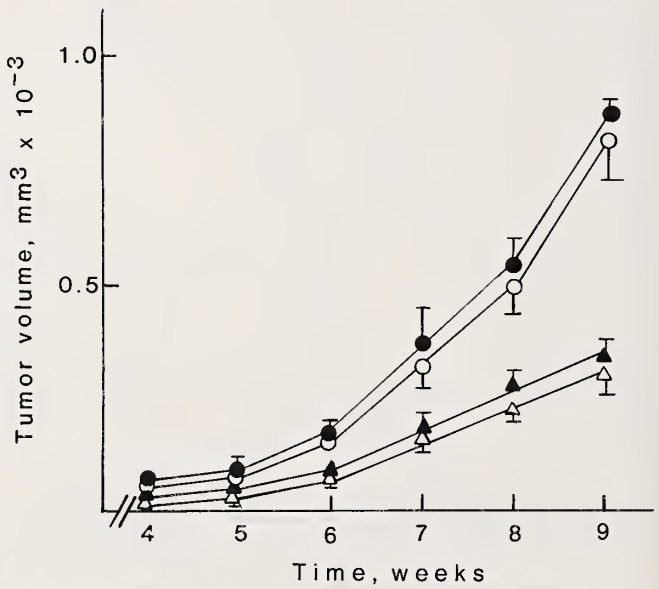

Fig. 5. Dose response curve for MAb 108 inhibition of tumor growth. NCI-H727 cells (10^7) were injected subcutaneously at day 1 and at day 2 PBS (●), 10 μg MAb 108 (○), 100 μg MAb 108 (▲), and 1 mg MAb 108 (△) injected intraperitoneally. Xenografts formed during week 3, and the tumor volume was determined weekly for weeks 4–9. The mean tumor volume ± SE of three determinations is indicated.

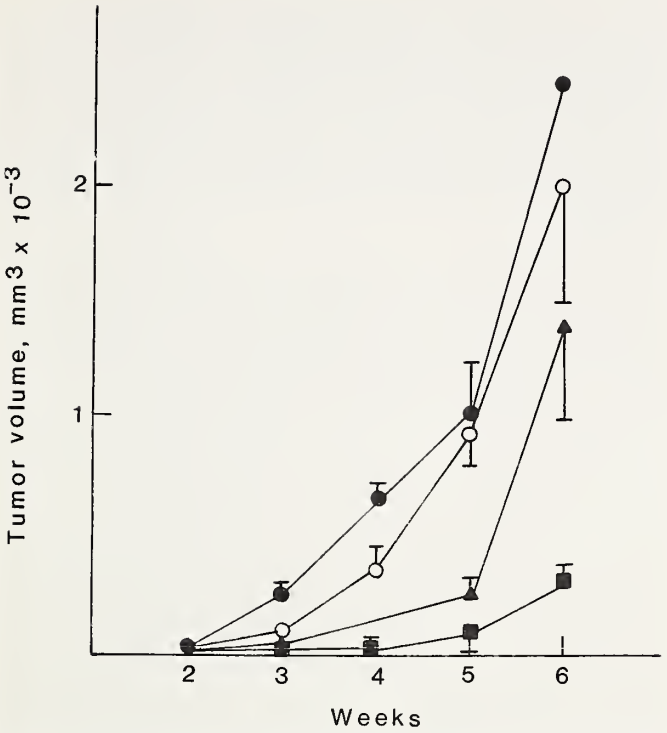

Fig. 6. Mode of administration of MAb 108 to inhibit tumor growth. NCI-H460 cells were injected subcutaneously at day 1. At day 2, PBS was injected in two animals (●, ▲) and 100 μg of MAb 108 injected in two animals (○, ■). In weeks 2–6, MAb 108 (100 μg) was injected three times weekly intraperitoneally in two animals (▲, ■). The mean value ± SE of three determinations is indicated.

the tumor volume was approximately 2500 mm^3. When MAb 108 (10 μg, intraperitoneal) was administered at day 2, tumor volume was decreased by approximately 20% in all succeeding weeks. When MAb 108 (100 μg) was administered three times weekly intraperitoneally after a palpable mass had formed, tumor volume was decreased by approximately 50%. When MAb 108 (100 μg) was administered 1 day after cell implantation and then injected three times weekly after a palpable mass had formed, tumor volume was reduced by approximately 90%. These data indicate that it is best to give MAb 108 1 day after cell implantation and to continue to administer it three times weekly until the termination of the study.

The specificity of the MAb was investigated in vivo. Fig. 7 shows that squamous cell carcinoma cell line NCI-H157 formed xenografts after 2 weeks and rapidly grew so that after 4 weeks the tumor volume was 2323 mm^3. When MAb (100 μg) was injected 1 day after cell implantation and injected three times weekly after a palpable mass had formed, tumor volume was reduced by approximately 65% when MAb 108 was administered but not when control antibody IgG was administered; the IgG control slightly stimulated tumor growth. When the tumors were excised, mice treated with MAb 108, but not with control antibody IgG, had significantly reduced tumor weight (Table 3). In contrast, the total body weight of the mice was not altered regardless of treatment (data not shown).

The localization of MAb 108 was also investigated. Table 4 shows that the density of ^{125}I-MAb 108 was approximately twofold greater in the tumor than in the lung, stomach, spleen, heart, or liver and approximately fivefold greater in the tumor than in the intestine. In contrast, control antibody IgG was preferentially localized to the lung (18.6 cpm per mg wet tissue) but not to the heart, tumor, stomach, spleen, liver, or gastrointestinal tract (<0.7 cpm per mg wet tissue). These data indicate the MAb 108 but not control antibody IgG associates with the tumor.

DISCUSSION

Previously, MAb 108 inhibited the effects of EGF on KB carcinoma and A431 cells, which have approximately 200 000 and 2 000 000 EGF receptors per cell respectively (*15,16*). In particular, MAb 108 inhibited high but not low affinity ^{125}I-EGF binding to A431 cells. Also, MAb 108 (30 nM) inhibited the autophosphorylation of the EGF receptor, phosphatidylinositol turnover, the elevation of cytosolic Ca^{2+}, and the elevation of cytoplasmic pH caused by 5 ng per ml EGF (*16*). MAb 108 inhibited the growth of KB tumor cell subcutaneous xenografts, prolonged the life span of animals with intraperitoneal tu-

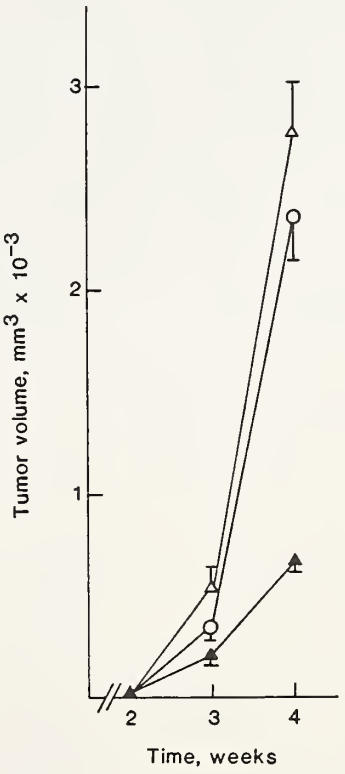

Fig. 7. Specificity of antibodies to inhibit tumor growth. At day 1, NCI-H157 were injected subcutaneously and at day 2 PBS (○), 100 μg MAb 108 (▲), and 100 μg of control IgG (△) were injected. In weeks 2–4, MAb was injected three times weekly intraperitoneally. The mean tumor volume ± SE of three determinations is indicated.

Table 3. Biochemistry of NSCLC xenografts*

Treatment	Tumor weight, g
PBS	2.46 ± 0.43
MAb 108	1.27 ± 0.07†
IgG	2.88 ± 0.34

*NCI-H157 tumors were dissected from nude mice at week 4 and weighed. The mean value ± SE of three determinations is indicated.
†$P < .05$.

mors, and reduced the number and size of experimental lung metastases (*15*). Here the effects of MAb 108 on lung cancer cell lines was investigated.

Previously, it was found that ^{125}I-EGF binding sites are present on NSCLC cell lines (approximately 100 000 per cell) whereas SCLC cells do not have detectable ^{125}I-EGF binding sites (*18*). Also, some NSCLC cells produce TGFα whereas most SCLC cell lines produce bombesin/gastrin-releasing peptide (*8,22*). Therefore, TGFα may be an autocrine growth factor for NSCLC.

Here ^{125}I-MAb 108 bound with high affinity (kd = 1.2 nM) to a class of sites on NSCLC-neuroendocrine cell line NCI-H460. Similar data were obtained using cell lines NCI-H727, NCI-H23, NCI-H1299, and NCI-H157. Also, MAb 108 bound with high affinity (kd = 2 nM) to KB cells (*15*). Because the number of ^{125}I-MAb and ^{125}I-EGF binding sites is similar, MAb 108 may bind to the EGF-R. Previously, we found that a 170-kd band was immunoprecipitated by MAb 108 (*18*). MAb 108 may not have, however, a total overlap with the EGF binding site. MAb 108 inhibited ^{125}I-MAb 108 binding to cell lines NCI-H23, NCI-H157, NCI-H1299, NCI-H157, NCI-H727, and EPLC-65H with IC_{50} values of 40 to 400 ng per mL but inhibited binding of ^{125}I-EGF with IC_{50} values of 300 to 3000 nM. Because MAb 108 is larger than EGF it may bind to more amino acid residues on the EGF-R than does EGF and only partially overlap the EGF binding site.

Because EGF stimulates the clonal growth of NSCLC cell line H157, it may function as an EGF receptor agonist. Previously, using Western blot techniques, we showed that EGF phosphorylated tyrosine amino acid residues of a 170-kd protein in NSCLC cell lines (*18*). Also, at 37 °C, ^{125}I-EGF was internalized by NSCLC cell lines. Here the clonal growth of several NSCLC cell lines was significantly inhibited by 1 but not 0.1 μg per mL MAb 108. MAb 108 may bind to the EGF-R and inhibit the ability of endogenous TGFα to stimulate NSCLC growth. Also, ^{125}I-MAb bound to the cell surface of NSCLC cell lines and was not readily internalized at 37 °C. These data suggest that MAb 108 functions as an NSCLC EGF-R antagonist.

Previously, MAb 108 (1 mg) or immunoglobulin molecule fragments Fab and $F(ab)_2$ inhibited the growth of subcutaneous KB carcinoma tumors when it was injected 1 day after tumor cell injection (*15*) whereas a MAb against DNP had no effect. For NSCLC cells, 100 μg of MAb 108 was approximately as potent at inhibiting NSCLC xenograft formation as 1 mg, whereas 10 μg was significantly less potent. Control antibody IgG slightly stimulated tumor growth. MAb 108 slowed the growth of NSCLC tumors when it was injected 1 day after the subcutaneous injection of cells. Also, MAb 108 slowed the growth of NSCLC tumors when it was injected three times weekly after a palpable mass had formed. MAB 108 was most effective at inhibiting tumor growth, however, when it was injected 1 day after cell injection combined with MAb 108 administration three times weekly. In general, the sooner MAb 108 was given after tumor cell injection, the more effective it was. This may be because the large MAb may have difficulty penetrating the interior of a bulky tumor. Because control IgG did not inhibit NSCLC growth, MAb 108 may inhibit NSCLC growth as a result of its interaction with the EGF-R.

Previously MAb 225 was localized to A431 and MDA468 cells but not to MCF-7 xenografts 1 week after injection (*23*). Here MAb 108 localized to the NCI-H157 tumors 1 week after injection into nude mice. The counts per minute of ^{125}I-MAb 108 per mg protein were approximately twofold greater and twofold lower in the tumor and intestine, respectively, relative to the lung, stomach, spleen, heart, or liver. In contast, counts per minute per mg protein of the control IgG was highest in the lung. MAb 108 may preferentially interact with the tumor due to its high concentration of the EGF-R. Similarly, MAb 108 interacted better with KB tumors relative to other organs. In KB tumors, MAb 108 was synergistic with cisplatin at inhibiting tumor growth (*15*). Also, a MAb 108 doxorubicin-conjugate inhibited KB carcinoma growth (*24*). It is not known whether these agents will inhibit NSCLC growth to a greater degree than does MAb 108.

In summary, MAb 108 inhibited EGF-R binding to NSCLC cells and growth of NSCLC cells in vitro and in vivo. Whether this agent will be useful in the treatment of NSCLC remains to be determined.

Table 4. Distribution of MAb 108 in nude mice*

Organ	Concentration
Tumor	4.56 ± 0.50
Lung	2.81 ± 0.05
Stomach	2.37 ± 0.49
Spleen	2.25 ± 0.15
Heart	2.10 ± 0.51
Liver	1.75 ± 0.01
Intestine	0.87 ± 0.08

*^{125}I-MAb 108 (10^6 cpm) was injected ip into nude mice bearing NCI-H727 tumors, and after 7 days the concentration of ^{125}I-MAb binding sites was determined (cpm/mg wet tissue). The mean value ± SE of three determinations is indicated.

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Epidermal Growth Factor Receptor as a Target for Therapy With Antireceptor Monoclonal Antibodies[1]

John Mendelsohn[2,3]

ABSTRACT—The epidermal growth factor (EGF) receptor is a potential target for antitumor therapy. Recent studies from many laboratories have found that this receptor is expressed in high levels on a variety of human tumor cells. Furthermore, the EGF receptor has been implicated in autocrine stimulation of cell growth in a number of experimental studies. We have produced anti-EGF receptor monoclonal antibodies (MAbs), which block the binding of EGF and transforming growth factor alpha (TGF-α), and can prevent ligand-stimulated activation of EGF receptor tyrosine kinase. These MAbs have been useful in studies of EGF receptor function. Experiments utilizing the MAbs to block ligand binding have demonstrated that autocrine stimulation of EGF receptor phosphorylation can occur via an extracellular pathway, involving TGF-α-mediated activation of EGF receptor on the surface of the cell. The capacity of anti-EGF receptor MAbs to inhibit cell proliferation has provided evidence of an autocrine stimulatory pathway in cultures of malignant human skin, breast, colon, and lung cells. Growth of a variety of human tumor xenografts can be inhibited in situations where autocrine dependency is demonstrable in cell culture. Imaging studies with anti-EGF receptor MAb labeled with indium 111 (^{111}In) demonstrated selective uptake in xenografts expressing high receptor levels. Based on these observations, a phase I trial was carried out with ^{111}In-labeled anti-EGF receptor MAb 225 IgG1 in patients with advanced squamous cell lung carcinoma, a tumor that invariably expresses large numbers of EGF receptors. In the case of squamous lung carcinoma, there is evidence that overexpression of EGF receptors correlates with worse clinical stage and worse prognosis. Escalating doses of 225 IgG1 were administered as a single intravenous injection over 1 hour. A dose of 120 mg of 225 IgG1 could produce saturating antibody levels in the serum for more than 3 days. This dose successfully imaged primary tumors as well as metastases greater than 1 cm in diameter, without producing toxicity. The tumor uptake determined by area-of-interest scanning was 3.4% of the injected dose, and there was considerable uptake in the liver. This study establishes the principle that anti-EGF receptor agents that block receptor function can be safely administered to patients and will localize, preferentially, to tumor cells bearing high levels of EGF receptors. Future studies will explore the potential therapeutic efficacy of repeated doses of anti-EGF receptor MAbs. [J Natl Cancer Inst Monogr 13:125–131, 1992]

The observations presented in this report draw upon the convergence of two major research themes: the potential of monoclonal antibodies (MAbs) as anticancer agents and the role of growth factors, in particular epidermal growth factor (EGF), in regulating cell proliferation.

RATIONALE: ANTIBODIES AGAINST RECEPTORS

The potential therapeutic uses of MAbs in the treatment of cancer have attracted considerable interest. Attention has been focused primarily upon two approaches: 1) serotherapy, which relies upon host immunological effector mechanisms to eliminate antibody-coated tumor cells, and 2) immunotoxin conjugates, which selectively target covalently bound drugs, toxins, or radionuclides to malignant cells. A third approach could utilize MAbs that act as pharmacologic agents by directly blocking biologic functions essential for cell proliferation. Receptors for essential growth factors are an ideal target for this approach because their location on the plasma membrane makes them readily accessible to antibody molecules.

Can an antireceptor antibody against a cancer cell reasonably be expected to produce stable changes in cell function? The answer appears to be yes on the basis of "experiments of nature" in which stable physiologic changes that result in disease are observed when patients develop antibodies against receptors. These antibodies bind to the acetylcholine receptor in myasthenia gravis (*1*), the thyroid-stimulating hormone receptor in forms of hyperthyroidism and hypothyroidism (*2*,*3*), and the insulin receptor in a rare form of insulin resistance that partially mimics diabetes mellitus (*4*).

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[3] These studies have been carried out in collaboration with investigators at the University of California, San Diego, and Memorial Sloan-Kettering Cancer Center. They include (chronologically) G. Sato, H. Masui, T. Kawamoto, D. Sato, J. Castagnola, G. Gill, C. MacLeod, H. Sunada, A. Goldenberg, S. Welt, F. Real, C. Divgi, S. Yeh, R. Gralla, M. Kris, S. Larson, and R. Kumar. Ms. Frantzie Paul provided expert typing and editing.

MAbs might interfere with cell proliferation and function by binding either to growth factors or to the receptors for these growth-promoting agents. Either approach has both theoretical appeal and experimental validation of potential efficacy. In favor of antibodies against receptors is the fact that the antibodies could act simultaneously through two mechanisms, by blocking receptor access to the required growth factor and by inciting an inflammatory response mediated by cells or complement, through recognition of the Fc portion of the immunoglobulin molecule. In addition, it is likely that the number of molecules of growth factor in the environment of a tumor greatly exceeds the number of receptor molecules on the tumor cells, providing a quantitative advantage for antibodies against the latter.

RATIONALE: GROWTH FACTOR RECEPTORS AS TARGETS

The rationale for selecting receptors as targets for anticancer therapy is compelling. Most cells require growth factors for proliferation, and depriving cells of a source of these factors (e.g., serum) typically results in growth arrest followed by cell death. The group or constellation of growth factors required by cells from different tissues varies enough to permit some selectivity in the application of antireceptor agents (*5*).

Could interruption in the activation of EGF receptors lead to a useful antitumor effect? Indirect evidence for this is provided by observations from a number of laboratories that, when the EGF receptor (*6,7*) and/or its ligand (*8,9*) is overexpressed in cells transfected with appropriate vectors, cells acquire transformed behavior in culture and in nude mouse xenografts. These experiments suggest that in certain experimental situations excessive activation of EGF receptors results in an altered phenotype typical of malignancy, raising the hope that interference with this pathway may modulate the growth of naturally occurring tumors.

Further support comes from the observation that many types of tumor cells display increased EGF receptors. Examples of such reports included cancer of the lung (*10–13*), glia (*14*), breast (*15*), head and neck (*16*), and bladder (*17*). In some cases, increased EGF receptor expression correlates with a poorer clinical outcome (*18*). The level of increased expression can reach an order of magnitude or greater (*19,20*). In these situations EGF receptors become tumor-specific, in the terminology used to describe antigens relatively overexpressed on malignant cells. Increased receptor content is often associated with increased production of transforming growth factor alpha (TGF-α) by the same tumor cells (*21*). This establishes conditions conducive to receptor activation by an autocrine stimulatory pathway.

Of course, the coexistence of EGF receptors and TGF-α in tumors does not prove that autocrine (or paracrine) stimulation of malignant cell proliferation is occurring. One aim of our research has been to utilize anti-EGF receptor MAbs as reagents to test for autocrine pathways in tumor cell cultures. Results of these studies are presented below.

PRODUCTION AND CHARACTERIZATION OF ANTI-EGF RECEPTOR MAbS

On the basis of the considerations discussed above, our laboratory has produced a panel of MAbs against the EGF receptor. The 225 IgG1 and the 528 IgG2a MAbs are similar in that they bind to the receptor with affinity comparable to the natural ligand (kd = 2n*M*) and compete with EGF binding. They can precipitate the receptor and block EGF-induced tyrosine kinase activity (*22–24*) (Fig. 1). In this experiment EGF-induced activation of receptor phosphorylation on tyrosine was blocked by a 15-fold excess of MAb 225. However, activation of a serine/threonine kinase (presumably secondary to stimulation of the receptor) was only partially inhibited by the excess MAb (Fig. 1, panel 4). The initial rates of internalization of EGF receptors into endosomes are the same in the presence of EGF or antireceptor MAb (*25*). However, subsequent receptor processing and catabolism appear to be through a slower pathway when the receptor is bound to the antibody, possibly related to differences in receptor tyrosine phosphorylation (*24*). It is likely that the MAbs do not react with the actual EGF binding site but react near enough to the binding site on the receptor to prevent EGF from binding since they react with a human-specific sequence and do not recognize EGF receptors on rodent cells.

The MAbs do not have intrinsic inhibitory activity against normal human cells in culture. However, they can block EGF- and TGF-α-induced stimulation of growth rate. This capacity has been demonstrated in cultures of human foreskin fibroblasts, which grow with a slow doubling time of 5–7 days in serum-free medium supplemented only with insulin and transferrin. The marked increase in proliferation rate that is induced by the addition of EGF can be blocked in a concentration-dependent manner by the concurrent addition of antireceptor MAb (*23*).

The inhibitory effects of 528 IgG1 or 225 IgG2a were explored with A431 human vulvar epidermoid carcinoma cells. The growth of these tumor cells, which express both EGF receptors and TGF-α in large quantities, was inhibited by anti-EGF receptor MAb (Fig. 2) (*23*). The lack of antiproliferative effect by MAb 455 is an important control because this antibody also binds to the EGF receptor but does not inhibit ligand binding and does not block activation of receptor tyrosine kinase. Thus, the specificity of MAbs for the EGF receptor is not sufficient to inhibit growth; only antibodies 225 and 528, which have the capacity to block EGF binding, can inhibit proliferation. This blocking activity could be reversed by the addition of carefully titrated amounts of EGF or TGF-α (*26*). The addition of ligand to antibody-treated cultures had to be titrated because excessive stimulation by EGF or TGF-α causes growth inhibition of A431 cells.

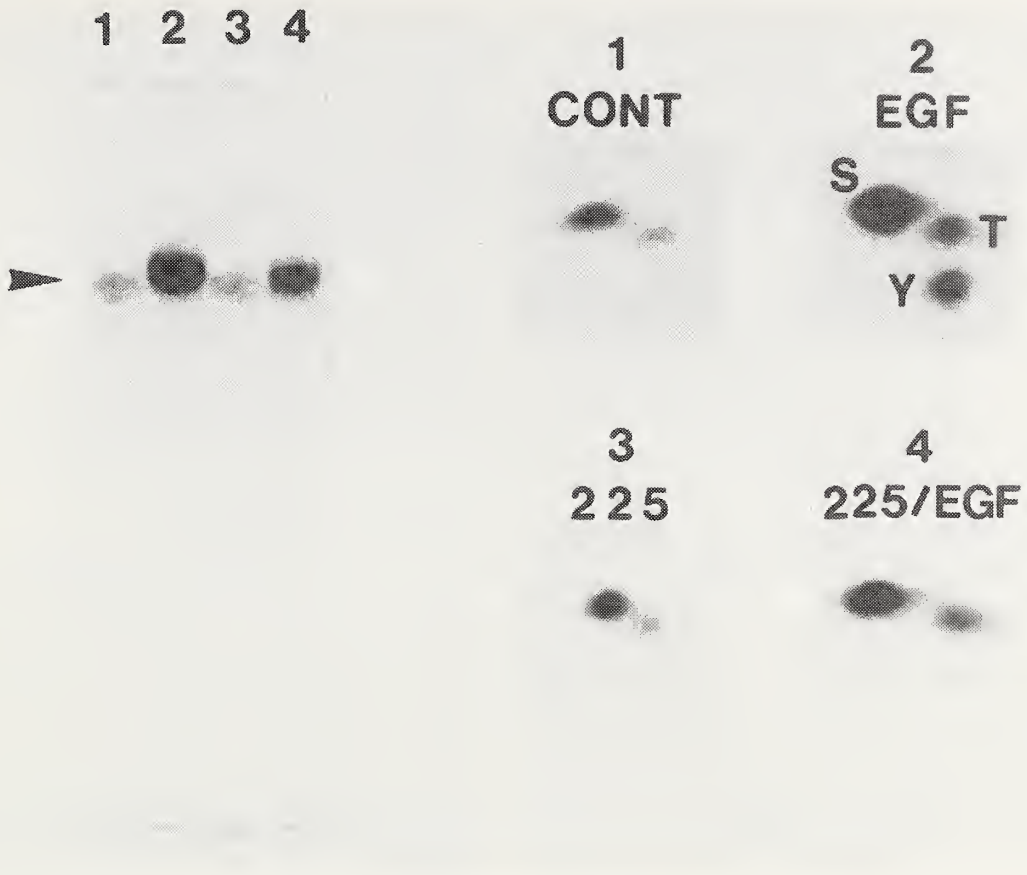

Fig. 1. Modulation of the EGF receptor phosphorylation by MAb 225. Near-confluent A431 cells prelabeled with ^{32}P-orthophosphate at 37 °C for 15 hours were incubated with 1) no addition; 2) 20 nM EGF (37 °C for 30 minutes); 3) 300 n*M* MAb 225 (0 °C for 30 minutes followed by 37 °C for 30 minutes); and 4) 300 n*M* MAb (0 °C for 30 minutes), then together with 20 n*M* EGF (37 °C for 30 minutes). To maintain consistency in the conditions, every culture was placed on ice for 30 minutes either in the presence or absence of MAb 225, prior to the addition of EGF. Left: autoradiography of immunoprecipitated ^{32}P-EGF receptor (arrowhead) separated in a 7% polyacrylamide gel in the presence of sodium dodecyl sulfate. Right: two-dimensional thin-layer electrophoresis patterns of ^{32}P-phosphoamino acids associated with the EGF receptor. S, phosphoserine; T, phosphothreonine; Y, phosphotyrosine. With longer autoradiography exposure time, baseline tyrosine phosphorylation in control cells becomes evident. (Figure courtesy of J Cell Physiol) (*24*).

USE OF ANTI-EGF RECEPTOR MAbS TO DEMONSTRATE AUTOCRINE STIMULATION

Although these results strongly suggested that the antibody acts by blocking binding of ligand to receptor on the cell's plasma membrane, we designed experiments to attempt to demonstrate formally that autocrine receptor activation does, in fact, occur on the external surface of the cell. The alternate possibility is that in cells producing both molecules activation of receptor by ligand could occur intracellularly, which would make it impossible to block receptor activation with an antibody. There is evidence that such an intracellular autocrine pathway occurs for cells bearing the receptor for platelet-derived growth factor and expressing that ligand (*27*).

When A431 cells were cultured under serum-free conditions, in the absence of exogenous ligand, EGF receptors were found to have a basal level of phosphorylation (*24*). When cells were labeled by culturing with ^{32}P-labeled phosphorus in the continuous presence of monoclonal antibodies that block binding of TGF-α to the EGF receptor, phosphorylation decreased to 30 ± 10% of the basal level (*28*). This reduction could not be accounted for by the decrease in receptor content attributable to down-regulation and catabolism of EGF receptors, which resulted from the binding of antireceptor monoclonal antibodies. The reduction in receptor phosphorylation mediated by the antibody was accompanied by accumulation of increased levels of secreted TGF-α species in the culture medium. We also pulse-labeled A431 cells for 15 minutes with [^{35}S]-cysteine, and immunoprecipitated the cell lysate with antiphosphotyrosine antibodies after various chase periods. The tyrosine-phosphorylated EGF receptor became detectable after 40 minutes of chase and reached a maximum after 4 to 6 hours; these times are in agreement with the intervals required for EGF receptors to reach the cell surface after synthesis and then to achieve maximal expression. In addition, only the 170-kd mature EGF receptor species, and not the 160-kd intracellular precursor, was immunoprecipitated with the antiphosphotyrosine an-

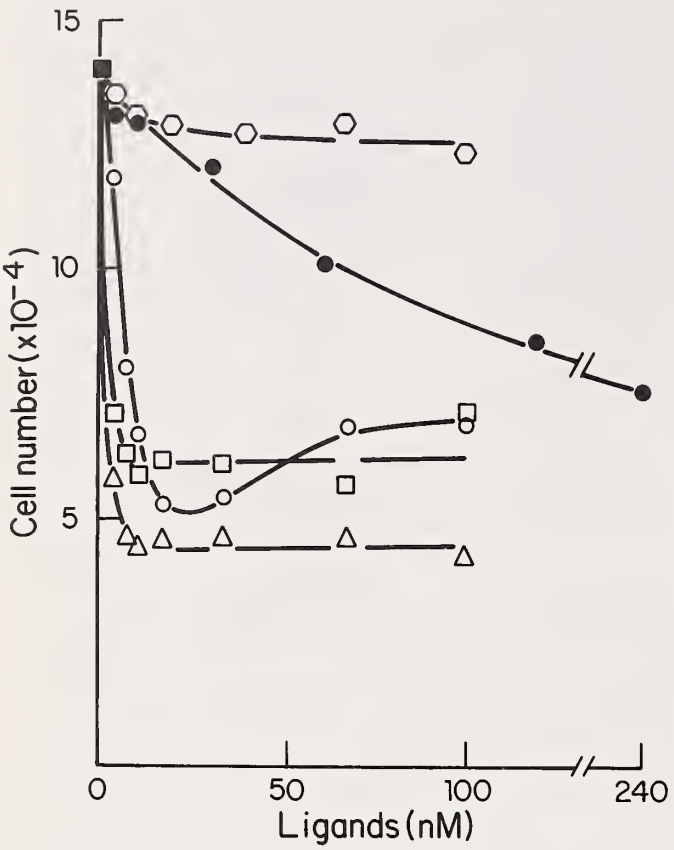

Fig. 2. Effects of monoclonal antibodies on A431 cell growth. A431 cells (1.5×10^4 per well) were incubated at 37 °C in DME-F12 medium with 528 IgG (○), 528 Fab fragments (●), 225 IgG (△), 579 IgG (□), 455 IgG (○). After 4.5 days the cells were trypsinized and counted. Each point represents the mean cell count of duplicate wells. (Figure courtesy of Mol Biol Med) (*23*).

tibody (*28*). The results of these pulse-chase experiments and the finding that the antireceptor MAb can block receptor phosphorylation suggest that activation of EGF receptors can result from the binding of an endogenous ligand (presumably TGF-α), which occurs at the cell surface and not during receptor biosynthesis or intracellular processing.

USE OF ANTI-EGF RECEPTOR MAbS TO EXPLORE AUTOCRINE PATHWAYS IN BREAST, COLON, AND LUNG CELLS

Recent studies with our blocking anti-EGF receptor antibodies have examined cells representing the three most common types of human cancer: breast (*29–31*), colon (*32,33*), and lung. In these studies, malignant or nonmalignant cell lines expressing EGF receptors and producing TGF-α were subjected to treatment in serum-free culture with antireceptor MAbs 225 or 528. Three types of response to the MAb were observed. In some cases the MAb could directly inhibit cell proliferation, and this inhibition could be reversed by addition of exogenous EGF to the culture; this suggests a requirement for autocrine, ligand-stimulated proliferation mediated by endogenous ligand acting extracellularly for optimal growth. In other cases, addition of a MAb to the culture had no direct effect on cell proliferation, but cell growth could be augmented by addition of exogenous EGF, and this augmentation was blocked by the antibody; this suggests that autocrine stimulation was not active in these cell lines, but the cell lines remained responsive to EGF. Finally, in still other cases the MAb did not alter growth rates in the absence or presence of exogenous EGF, suggesting that in these cells the presence of EGF receptors was irrelevant to their proliferation in culture. The results indicate that, as with other potential anticancer therapies, not all cells will respond to an anti-EGF receptor agent.

PRECLINICAL STUDIES

In vivo effects of treatment with anti-EGF receptor MAbs were assayed against xenografts of human tumor cells. A treatment schedule of intraperitoneal injections twice weekly was selected, based on a measured MAb half-life in serum of 3 days. Antitumor activity was most prominent when the malignant cells expressed high levels of EGF receptors and responded to TGF-α in culture. Administration of either 225 IgG1 or 528 IgG2a MAb intraperitoneally, beginning concurrently with tumor cell implantation subcutaneously, caused a dose-dependent inhibition of A431 squamous tumor cell growth (*34*). Administration of 2 mg twice weekly resulted in complete suppression of tumor growth, which persisted for 3 months after completion of a 3-week course of therapy. Comparable inhibition of xenograft tumor growth was observed with MDA-468 breast carcinoma (Fig. 3) (*35*) and DiFi colon carcinoma (Masui H, Mendelsohn J, Boman B, unpublished observations). Antitumor effects were not observed against other tumor cell lines that were not inhibited by MAbs in culture (*34*). An $F(ab')_2$ fragment administered in vivo could produce antitumor activity, although requiring higher concentrations than needed with a complete antibody (Masui H, Mendelsohn J, unpublished observations). The observation that a number of EGF receptor-bearing tumor xenografts were not inhibited by MAbs suggests that the antibodies were active against a subclass of tumors with particular susceptibility to blockage of the receptor. We postulate that the characteristic defining the susceptible subclass of tumor cells is the production of TGF-α and response to it in an autocrine fashion (*36*). It should be noted that the evidence for physiologic effects of anti-EGF receptor MAbs does not rule out the possible concurrent activity of these antibodies as immune effector agents.

Another set of experiments demonstrated that anti-EGF receptor MAb 225 IgG1 could selectively image tumor xenografts bearing high levels of EGF receptors (*37*). These studies were performed with MAb conjugated with Indium 111 (^{111}In). Further in vivo studies were performed with chimpanzees after it was demonstrated that the anti-

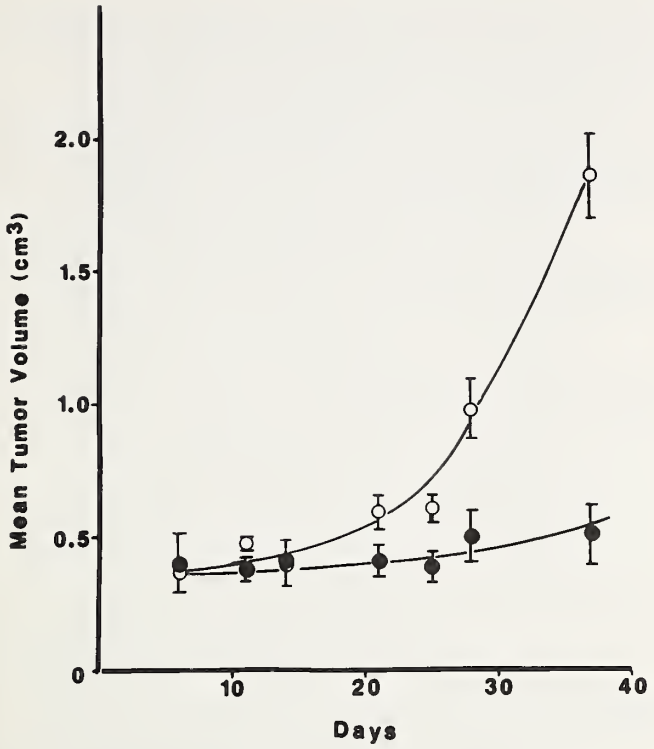

Fig. 3. Antitumor activity of 528 IgG2a against xenografts of MDA-468 human breast adenocarcinoma cells. MDA-468 cells (10^7) were injected subcutaneously into groups of six mice. For treatment, 2 mg of 528 IgG2a was injected intraperitoneally on the day of tumor cell inoculation and twice weekly thereafter. (○) MAb treated; (●) control. (Figure courtesy of Cancer Cells) (*35*).

EGF receptor MAbs bind to their tissues. No toxicities were observed following administration of MAbs in multiple IV injections reaching 650 mg total dose (Hybritech Inc., unpublished observations).

PHASE I CLINICAL TRIAL WITH ANTI-EGF RECEPTOR MAb

For our first clinical trial with anti-EGF receptor MAbs we selected patients with advanced squamous cell carcinoma of the lung. This tumor has been demonstrated to express high levels of EGF receptors in a large number of studies (*10,11*). Furthermore, high levels of receptor expression correlate with more advanced clinical stage and histopathology (*12*) as well as with a worse prognosis (*13*).

A phase I trial of anti-EGF receptor MAb therapy was initiated with 225 IgG1, which is less likely to activate complement and inflammatory mechanisms than is 528 IgG2a (*38,39*). By selecting 225 IgG1 for our clinical trial, we were able to design a more pure test of the hypothesis that an antibody can exert antitumor effects in vivo by directly affecting a physiologic function of the antigen, in this case the EGF receptor. The goals of this phase I trial were to define the toxicity and pharmacokinetics of ^{111}In-225 IgG1 in patients and to determine if ^{111}In-225 IgG1 localizes to sites of squamous carcinoma of the lung.

Table 1. Tumor visualization with ^{111}In-225*

	>40 mg MAb	All doses MAb
No of patients	10	16
Primary lesions		
Imaged	9	12
Observed by CT, x ray	9	14
Presumed metastases >1 cm		
Imaged	8	8
Observed by CT, x ray	8	13
Presumed metastases <1 cm		
Imaged	0	0
Observed by CT, x ray	1	5

*Derived from ref. *39*.

Eligible patients had stage III or stage IV squamous carcinoma of the lung, with a performance status greater than 60% (Karnofsky scale). They were first offered treatment and/or given treatment with courses of radiation or chemotherapy. Patients in groups of three received 1 hour infusions of the MAb. The first group received 1 mg of unlabeled MAb as a single injection. Subsequent groups received 4 mg of MAb labeled with 5mCi ^{111}In, mixed with 16, 36, 116, or 296 mg of unlabeled MAb.

We have not observed any toxicity in patients treated with up to 300 mg of anti-EGF receptor MAb. Imaging studies on patients who received 40 mg of MAb or more revealed visualization of each primary tumor and could detect by CT scan or x ray all but one of the presumed sites of metastatic disease with a diameter of more than 1 cm (Table 1, Fig. 4). The percentage of injected dose in the

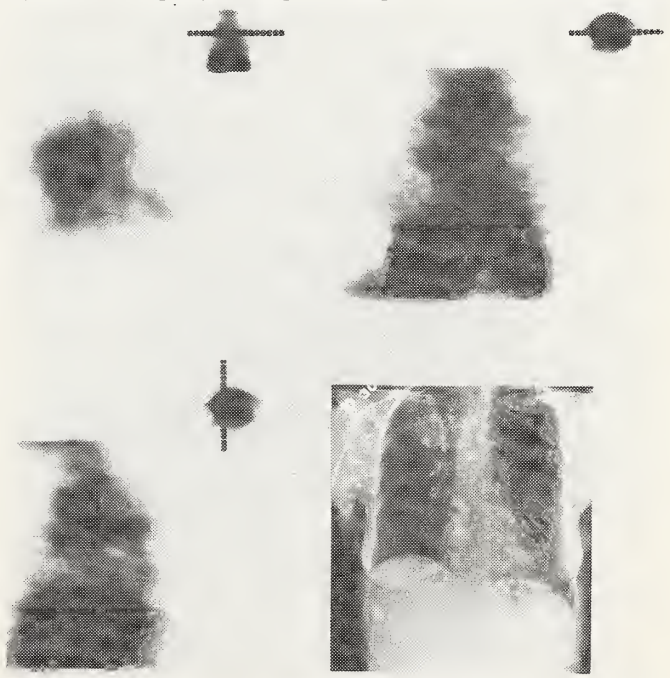

Fig. 4. Unmodified thoracic SPECT images of a patient with a right lung lesion, taken 72 hours after administration of 120 mg of antibody. Clockwise from upper left: transverse reconstruction, coronal reconstruction, chest x ray, and sagittal reconstruction. Transverse slice is at the level of the primary lung lesion. Reference planes for each of the slices are on the upper right. (Figure courtesy of J Natl Cancer Inst) (*39*).

tumor at 72 hours, determined by area-of-interest scanning with a gamma camera, was 3.4% in patients who received 120 mg of MAb. Liver uptake of labeled MAb was 10-fold greater. This may be due to the presence of EGF receptors on the large number of hepatocytes and/or to the well-described capacity of parenchymal and phagocytic cells in the liver to ingest both MAbs and labeled indium. The serum concentration of ^{111}In-225 IgG1 was more than 40 nM for more than 3 days, when the administered dose was escalated to 120 mg and higher. This level of MAb could saturate EGF receptors if this level were achieved in tissues. All patients produced human antimouse antibodies.

These observations suggest that 225 IgG1 may be useful for imaging tumors that bear increased numbers of EGF receptors, and they indicate that therapy with antireceptor MAbs, or with immunoconjugates of the MAbs, are worthwhile areas for clinical investigation in the future. The data indicate that patients tolerated the presence of saturating concentrations of an EGF receptor-blocking agent in their blood for a period of more than 3 days without side effects. Our studies with athymic mouse xenografts suggest that therapeutic intervention with an antireceptor MAb will require prolonged exposure of tumor cells to a blockade. Studies are planned with multiple doses of hybrid MAbs containing human constant regions. Production of small polypeptide analogues that bind to and block receptors could provide more effective antitumor agents in the future.

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Systematic Development of Bombesin/Gastrin-Releasing Peptide Antagonists[1]

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ABSTRACT—Several families of very potent bombesin (Bn) receptor antagonist analogues have recently been developed and their biological potencies evaluated in a number of in vitro systems including guinea pig and rat pancreatic acini and Swiss 3T3 cells. These studies showed that analogues can exhibit diverse properties ranging from full antagonists, partial agonists, or full agonists depending on the assay system and animal species employed. We have developed two classes of more potent, shorter chain antagonists based on [ψCH_2NH(13-14)]Bn(6-14) and desMet^{14}Bn(6-13)NH_2 structures. [D-Phe^6ψ Leu^{13}-Leu^{14}] Bn(6-14)NH_2 was a potent antagonist (K_i 6nM) in Swiss 3T3 cells and guinea pig acini but exhibited 10% partial agonist activity and lower binding affinity (K_i 60 nM) in rat acini. The partial agonism could be eliminated by using p-Cl-Phe or D-Phe at the C-terminus and partially eliminated using D-4-Cl-Phe in position 6. With the antagonist [D-Phe^6]Bn(6-13)NH_2 (K_i 96 nM), alkyl substituents on the amide group increased affinity 25-fold with the propylamide being the most potent peptide (K_i 4 nM) in 3T3 cells or guinea pig acini. It did, however, have high 40% partial agonist activity in rat acini. Alkyl esters or hydrazide derivatives were, in contrast, pure antagonists in all systems tested with [D-Phe^6]Bn(6-13)OMe having the highest affinity in all systems and also excellent in vivo properties. All of the potent antagonists examined had little affinity for neuromedin B—preferring bombesin receptors, which had entirely new ligand structure-activity relationships. The similar effects created by position 6 and 14 side-chain modifications on antagonist/agonist activity has suggested a possible interaction between these positions in the Bn receptor-bound conformation. With this in mind, cyclic analogues were synthesized with covalent bridges between these positions. [D-Cys^6,D-Ala^{11}, Cys^{14}] Bn(6-14) retained significant binding affinity (K_i 64 nM). The D-amino acid residues were instrumental in creating this degree of affinity and this appears to confirm that Bn/gastrin-releasing peptide (GRP) adopts a folded configuration in its receptor-bound state. Thus, there is now an excellent possibility of developing potent conformationally restricted Bn agonists and antagonists that would be of value in computer modeling and physicochemical studies. The inhibitory effects of some of the antagonist analogues on human small cell lung tumor growth have been examined, and some favorable results were obtained with those tumors demonstrated to possess Bn receptors. [J Natl Cancer Inst Monogr 13:133–139, 1992]

Bombesin (Bn) (*1*) and its mammalian counterpart, gastrin-releasing peptide (GRP) (*2*), have amino acid sequences that are closely related in their C-terminal regions (Fig. 1). They share a wide range of CNS and peripheral biological activities that have been recently reviewed (*3*). Notable among these activities are increased grooming (*4*), decreased food intake (*5*), and altered hormone secretion (*6*) after central injection and stimulation of gastrin and gastric acid release (*7*) and pancreatic amylase secretion (*8*) after peripheral injection. Bn/GRP immunoreactivity is widely distributed throughout the gastrointestinal tract (*9*), the brain (*10*), and lungs (*11*). Specific binding sites for the peptides have been characterized on pancreatic acinar cells (*12*) and in the rat brain (*13*), where the distribution is markedly similar to substance P. GRP was isolated (*14*) from porcine nonantral gastric tissue and was found to be a 27-residue peptide, the C-terminal seven amino acids of which are identical to bombesin. The known biological activities of GRP are thus far identical to bombesin.

Minamino et al. (*15,16*) also isolated two peptides, named neuromedins B and C, from pig brain and spinal fluid. Neuromedin C comprises the C-terminal 10 amino acids of GRP whereas neuromedin B contains a leucine (Leu) replacement for glutamine (Gln) in position 7, threonine (Thr) for Val-10, and phenylalanine (Phe) for Leu-13 (Fig. 1) and thus has a distinct homology to the amphibian peptide, litorin. Differences in the amino acid sequences of peptides within the same family have now led to the discovery of numerous instances of receptor subtypes with distinct ligand binding specificities—for instance, CCK/gastrin, VIP/secretin/glucagon, and the opiate peptides come to mind, and a similar situation has now been found to exist for Bn/neuromedin B (NMB) with the discovery (*17*) of NMB specific receptors in rat esophageal

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cells. NMB receptors have now been shown to be the most widespread receptor for Bn peptides in the central nervous system and in numerous gastrointestinal smooth muscles (*18–22*). It appears that important new physiological neuromodulatory functions for NMB, some of which were perhaps previously attributed to Bn/GRP, will be found. We have also made significant progress in designing peptides highly specific for either Bn or NMB receptors, and this will be discussed later.

Bn/GRP also has mitogenic activity on several types of tissues and cells, including bronchial epithelial cells (*23*), human small-cell lung cancer (HSCLC) cells (*24,25*), and murine Swiss 3T3 cells (*26*), and GRP appears to be an important autocrine growth factor for HSCLC thus providing the rationale for the development of competitive receptor antagonists. Bn production from carcinogen-induced rat hepatocellular tumors has also been reported (*27*). Bn receptors have also been found on PC3 and PMU23 human prostate tumor cells, and Bn stimulated the growth of these cells in culture (*28*). GRP receptors have also just been reported to be present in some human breast cancer cell lines (*29*) but not in normal breast tissue, which suggests that Bn antagonists should be tested for inhibition of breast tumor growth also. Receptors have been reported (*30*) on mouse colon MC-26 cancer cells, and bombesin had a significant growth-promoting effect on these cells in vitro. Recent reports of receptor down-regulation in pancreatic islet cells (*31*) and Swiss 3T3 cells (*32*) suggest the possible use of Bn/GRP agonist analogues for inhibition of cell growth and, indeed, Bn itself has been reported (*33*) to inhibit the growth of human pancreatic adenocarcinoma in nude mice. The use of Bn antagonists as preventive agents in precancerous states where elevated Bn levels are found is suggested by increased levels of these peptides in asymptomatic cigarette smokers (*34*) and in breast cyst fluid (*35*) (breast cysts are linked to much-enhanced rates of breast tumors).

The first progress in the search for competitive receptor antagonists of Bn/GRP was made in 1984 when Jensen et al (*36*) reported that analogues in the "spantide" series of substance P antagonists (for instance, D-Arg-Pro-Lys-Pro-Gln-Gln-D-Trp-Phe-D-Trp-Leu-LeuNH_2) were also able to bind to bombesin receptors on pancreatic acini and function as weak bombesin antagonists with IC_{50}s in the μM potency range. However, their lack of specificity and their low potency has severely limited their usefulness. We discovered a new series of antagonists based on the replacement of histidine (His)[12] in bombesin with a D-Phe residue alone (*37*) or, preferably, in combination with a D-Phe residue in position 6 (*38*). These also displayed relatively low binding affinity and have been of little practical value. Since then, two classes of extremely potent yet structurally simplified Bn analogues have been developed.

PEPTIDES CONTAINING A PSEUDOPEPTIDE BOND

A new design strategy of peptide bond rather than amino acid side-chain alteration yielded (*39*) the first really potent bombesin antagonist, [Leu[14],ψ13-14]-bombesin (Fig. 1 and Table 1) in which the CONH peptide bond between positions 13 and 14 was replaced with a CH_2NH group (often called a reduced peptide bond). This peptide exhibited an IC_{50} and K_i of 35, 60, and 20 nM, respectively, for inhibition of Bn-stimulated amylase release from and binding to guinea pig pancreatic acini cells and inhibition of growth of 3T3 cells, respectively. It has been used extensively and successfully as a general and specific Bn antagonist by many investigators. Replacement of the 26–27 CONH group with an ether CH_2O linkage also resulted in a potent antagonist in Ac-GRP(20–27), (*40*).

Elimination of N-terminal amino acids from the [Leu[14],ψ13-14]-bombesin pseudotetradecapeptide resulted in considerable loss of antagonist activity and binding affinity in all systems, including Swiss 3T3 cells (Table 1) where [Leu[14],ψ13-14]-bombesin(6–14) only had a K_i of 291 nM. However, the presence of a D-aromatic amino acid in the 6 position allowed five of the N-terminal amino acids to be removed while actually increasing antagonist affinity to 7 nM. Many other combinations of aromatic amino acids in positions 6 and 14 gave analogues that

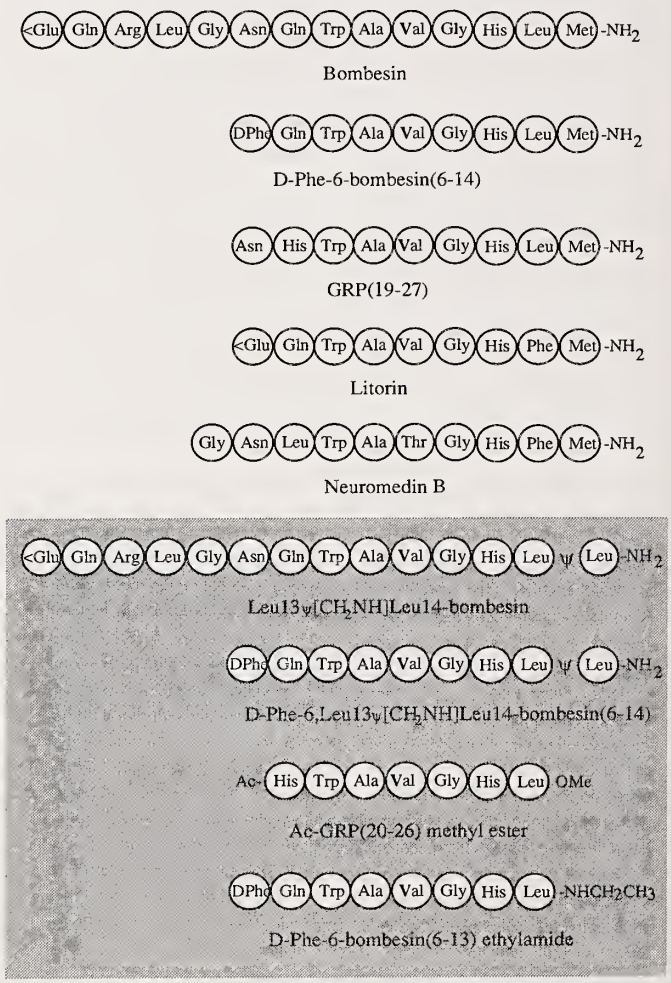

Fig. 1. Typical bombesin/GRP agonist and antagonist (shaded) structures.

Table 1. Bombesin ψ-analogue inhibition of ^{125}I-[Tyr^4]bombesin binding to Swiss 3T3 cells

ψ-Analogue	K_i (nM)
Leu^{14},ψ13-14-Bn	65
D-Ala^{11},Leu^{14},ψ13-14-Bn	67
D-Phe^{11},Leu^{14},ψ13-14-Bn	222
D-Ala^5,Leu^{14},ψ13-14-Bn	75
Phe^{14},ψ13-14-Bn	7
Phe^{13},ψ13-14-Bn	32
D-Phe^6,Leu^{14},ψ13-14-Bn	73
Leu^{14},ψ13-14-Bn(5-14)	123
Leu^{14},ψ13-14-Bn(6-14)	291
ψ8-9-litorin	10
D-Phe^6,Leu^{14},ψ13-14-Bn(6-14)	7
D-Nal^6,Leu^{14},ψ13-14-Bn(6-14)	14
D-Phe^6,Phe^7,Leu^{14},ψ13-14-Bn(6-14)	154
D-Phe^6,Phe^{14},ψ13-14-Bn(6-14)	3
D-Phe^6,Val^{13},Leu^{14},ψ13-14-Bn(6-14)	5
D-Phe^6,4-Cl-Phe^{14},ψ13-14-Bn(6-14)	5
D-Phe^6,D-Phe^{14},ψ13-14-Bn(6-14)	3
D-4-Cl-Phe^6,Phe^{14},ψ13-14-Bn(6-14)	6
N-Me-D-Phe^6,Phe^{14},ψ13-14-Bn(6-14)	4
D-Nal^6,Phe^{14},ψ13-14-Bn(6-14)	15
D-Nal^6,Nal^{14},ψ13-14-Bn(6-14)	70
ψ9-10-neuromedin B	1012

either approached or slightly exceeded these binding levels (Table 1). These analogues retained high specificity for Bn receptors and were also potent inhibitors of Bn-stimulated amylase release from guinea pig and rat pancreatic acinar cells and the growth of Swiss 3T3 cells as measured by ^{3}H-thymidine incorporation (*41*).

DESMET^{14}-BOMBESIN ANALOGUES

A different method of producing peptide antagonists involving modification of the C-terminus by an approach originally devised by Martinez and Bali (*42*) and Martinez et al (*43*) for cholecystokinin/gastrin peptides was also successfully applied to GRP (*44–46*) and bombesin (*44,45*). The alterations generally involved removal of the C-terminal methionine (Met) residue from either GRP (*20–27*) to produce an initial weak competitive antagonist (*44*) or from a series of Bn analogues (*47,48*) within which [D-Phe^6]-bombesin(6-13)NH_2 had a K_i of 23 nM for Swiss 3T3 cells (Table 2). Subsequently, potencies of these octapeptides were increased further by adding suitable alkyl substituents to the C-terminal amide group (*45–47*). These have included ethyl (K_i 3 nM), propyl (K_i 2 nM), and higher alkyl groups such as butyl (K_i 5 nM) and hexyl (K_i 6 nM). Interestingly, the higher alkylamides began to show partial agonist activity on guinea pig acini and Swiss 3T3 cells at the higher dose levels (*47*). Other C-terminal groups were also extremely effective, with hydrazides and phenethyl, methyl, and ethyl esters being among the best. The methyl ester analogue (K_i 0.3 nM) was the most potent analogue in a number of biological systems (*22*) and was found (*49*) to have particularly good pharmacokinetic properties during inhibition of Bn-stimulated amylase release in the rat. [D-F_5Phe^6,D-Ala^{11}] Bn(6-13) OMe (Table 2) was another analogue with particularly high affinity and also resistance to enzymatic degradation and is a likely candidate for in-depth study for effects on tumor growth. Replacement of the C-terminal dipeptide with statine and statine-related structures also gave analogues with interesting levels of antagonist potency (*50*).

Table 2. Bombesin [desMet^{14}]-analogue inhibition of ^{125}I-[Tyr^4]bombesin binding to Swiss 3T3 cells

Analogue	K_i (nM)
Bn(1-13)NH_2	18
Bn(6-13)NH_2	1216
D-Phe^6-Bn(6-13)NH_2	23
D-Phe^6,Sar^{11}-Bn(6-13)NH_2	64
D-Nal^6-Bn(6-13)NH_2	28
D-Nal^6,Ala7-Bn(6-13)NH_2	201
D-Phe^6-Bn(6-13)OH	1377
Bn(6-13) ethylamide	31
Ac-Bn(7-13) ethylamide	18
D-Phe^6-Bn(6-13) ethylamide	3
D-Phe^6-Bn(6-13) propylamide	1.7
D-Phe^6-Bn(6-13) butylamide	5
D-Phe^6-Bn(6-13) hexylamide	6
D-Phe^6-Bn(6-13) heptylamide	11
D-Phe^6-Bn(6-13) phenethylamide	18
D-Phe^6-Bn(6-13) OMe	0.3
D-F_5Phe^6,D-Ala^{11}-Bn(6-13) OMe	0.2
D-Phe^6-Bn(6-13) OEt	1
Ac-GRP(20-26) OEt	2
D-Phe^6,D-N-Me-Ala^{11}Bn(6-13) OMe	8
D-Phe^6-Bn(6-13) $NHNH_2$	2
D-Phe^6-Bn(6-13) NHNHMe	3
D-Phe^6-Bn(6-13) $NHNMe_2$	451
D-Phe^6-Bn(6-13) NHNHtBu	214

RESIDUAL PARTIAL AGONIST ACTIVITY IN BOMBESIN ANALOGUES—DEVELOPMENT OF MORE GENERAL ANTAGONISTS

As already mentioned, several of the higher alkylamide des-Met^{14} analogues of Bn displayed significant agonist activity on guinea pig acini and murine Swiss 3T3 cells. Even our original antagonist, [Leu^{14},ψ13-14]-Bn, was shown to have agonist activity in a frog esophageal peptic cell system (*51*). We have recently found (*52,53*) that the situation becomes even more complex when using rat pancreatic acini cells as an assay system whereupon a far greater proportion of peptides in all classes display significant partial agonist properties. Alkylamide derivatives with groups higher than an ethyl group were partial agonists in the rat (*52*), as was the phenethylamide. It is not known why the longer alkyl side chains promote agonist activity, although one can speculate that groups beyond a certain length may be mimicking the side chain of the position 14 amino acid. Even more puzzling was the destruction of all agonist activity in the phenethylamide

analogues simply by substitutions on the benzene ring (*52*). The unsubstituted amide, hydrazide, methyl hydrazide, 2,2-dimethyl hydrazide, *t*-butyl-hydrazide, and methyl and ethyl esters were devoid of any detectable agonist properties. In the guinea pig, only the butylamide and hexylamide peptides displayed partial agonist properties so that the rat appears to have a much increased sensitivity toward receptor activation and consequently to discriminate more poorly between the various ligand types (*52*).

In the reduced peptide bond series of antagonists the situation was found (*53*) to be even worse with most peptides, including the original member of the series, [Leu^{14},ψ13-14]-Bn, which had partial agonist activity in the rat pancreatic acini cells. Partial agonist potency was on the order of 7% maximal with this analogue but rose to 40% with [D-Phe^{6},Phe^{14},ψ13-14]-Bn(6-14), the presence of a C-terminal aromatic side-chain being particularly prone to causing receptor activation in the rat. On the other hand, none of the analogues exhibited detectable agonist activity on either guinea pig acini or Swiss 3T3 cells.

As with the desMet^{14}-analogues, general pure antagonist analogues in the reduced peptide bond family could also be produced (*53*). Surprisingly, substitution of a Cl on the aromatic ring of the [D-Phe^{6},Phe^{14}, ψ13-14]-Bn(6-14) analogue completely eliminated rat partial agonist activity. Rat agonist activity was also eliminated by the use of a D-Phe^{14} residue in [D-Phe^{6},D-Phe^{14},ψ13-14]Bn(6-14), although this analogue was not as potent as the 4-Cl-Phe^{14} analogue. Several very potent analogues with much simplified structures have, therefore, been developed and behave as pure antagonists in all systems thus far examined.

SPECIFICITY FOR THE Bn OR NMB RECEPTOR

Von Shrenk's development (*54*) of a specific ligand binding and bioassay system for this receptor which utilizes rat esophageal muscle cells has opened up an important new area of Bn analogue research. As can be seen in Table 3, these cells much prefer neuromedin B to Bn-like peptides—quite the reverse of the rat acinar cells. Many of the analogues just discussed have been examined in this system, and the results are shown in Table 3 in comparison to the rat acinar cell data. In general, all of the potent Bn antagonists have very low affinity for NMB receptors and neither the ψ-bond replacement or des-Met^{14} deletion strategies produce really useful NMB antagonists. Strangely enough, the best NMB antagonist is a [D-Phe^{12}]Bn analogue. This could be an important lead for NMB antagonist design. The marked difference in NMB and GRP affinities for these two receptor types suggests that even higher affinities and selectivities will be achieved with analogues in the future. The development of highly selective agonists or antagonists is clearly needed in order to elucidate the physiological roles of NMB and the development could assume therapeutic importance if NMB receptors are discovered on tumor cells thus indicating a possible mitogenic response to this peptide also. The mapping of the comparative distributions of GRP and NMB receptor populations will be greatly aided by the recent isolation and cloning of the two receptors (*55*,*56*).

CYCLIC Bn ANALOGUES

A useful approach with other peptides, for instance, somatostatin, enkephalin, and α-MSH, has been the introduction of additional conformational restriction into analogues by covalent cyclization. This enables far more meaningful stereochemical information to be obtained from computer molecular modeling and solution 2D NMR experiments. Work in our laboratories is already proceeding along these lines, and some progress has already been made in delineating suitable covalent cyclization points in the Bn(6-14) nonapeptides. Position 6 was chosen because of the efficacy of D-amino acids present at this point in both agonists and antagonists and the possibility that this could provide a folding point (in addition to Gly-11) in a cyclic structure. The analogue in Fig. 2 contains a D-Cys residue in position 6 and L-Cys in position 14, which were

Table 3. Comparison of bombesin/NMB analogue binding to rat pancreatic acini and esophageal cells

Analogue	K_d, pancreas, n*M*	K_d, esophagus, n*M*	Ratio K_d pancreas/esophagus binding
Bn	4	2	2
GRP	15	30	0.5
NMC	20	36	0.55
NMB	351	0.3	1170
Litorin	5.7	0.07	81
Spantide	13 000 (antag)	4400 (antag)	3
D-Phe^{12},Leu^{14}-Bn	1300 (antag)	9134 (antag)	0.14
D-Phe^{5,12},Leu^{14}-Bn	>10 000	586 (antag)	>17
D-Phe^{6,12},Leu^{14}-Bn	>10 000	3136 (antag)	>3
Leu$^{13}\psi$Leu^{14}-Bn	434 (antag)	44 000 (no activity)	0.01
D-Phe^{6},Leu$^{13}\psi$Cpa^{14}-Bn	42 (antag)	5800 (no activity)	0.007

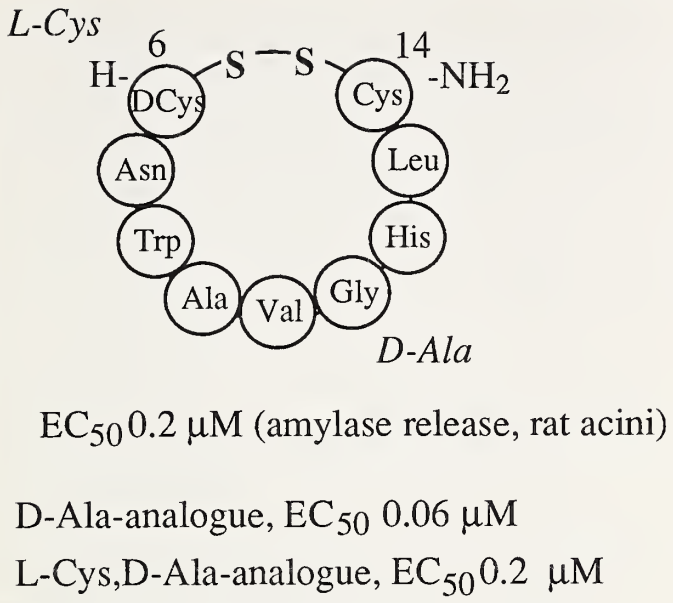

Fig. 2. Cys-bridged cyclic Bn analogues that retain significant amylase releasing activity on rat pancreatic acinar cells. Note the increased activity generated by D-amino acid substitutions.

utilized for disulfide bridge formation in the usual fashion. This peptide displayed significant agonist potency with a K_d of about 0.2 μM (about 1% of the affinity of Bn itself), which was increased almost 10-fold by inclusion of the D-Ala residue (Fig. 2). L-Cys in the first position of this analogue resulted in about a 10-fold loss of potency. These results provide numerous exploitable insights into the conformational properties of receptor-bound Bn—for instance, this is the first direct evidence that Bn is indeed folded, probably in a type II β-turn around Gly when bound to its receptor.

ANTI-TUMOR PROPERTIES OF Bn ANTAGONISTS

A considerable amount of work has been done on the effects of our original Bn antagonist on HSCLC growth in vitro and also on transplanted tumors from various human strains (*57,58*). This has just been extended to some of the newer analogues (*59*) where good correlation was found between binding affinities on acinar and HSCLC cells. In our own and collaborative studies, progress was slowed by the discovery of the partial agonist activity of some of the analogues chosen for the tumor growth studies. However, a study was recently completed using [D-Phe^6,ψLeu^{13}-Cpa^{14}]Bn(6-14) (pure antagonist), [D-Phe^1,Leu^{8,9}]litorin (pure agonist), and [D-Cpa^6,ψLeu^{13}-Phe^{14}]Bn(6-14) (mixed agonist/antagonist). The tumor types screened were NCI-H345 (shown to have Bn/GRP receptors) and NCI-H69 (no detectable Bn/GRP receptors) transplanted into athymic nude mice. The results are summarized in Table 4. With the former, both antagonists were able to partially inhibit tumor growth with the pure antagonist giving the greatest inhibition, as expected. However, the potent agonist, [D-Phe^1,Leu^{8,9}]litorin, was also able to partially prevent tumor growth, suggesting various mechanistic possibilities such as Bn receptor down-regulation as already discussed. Totally unexpected was the stimulatory effect of both the pure antagonist and mixed antagonist/agonist on NCI-H69 tumor growth because these cells lacked detectable Bn receptors. Even if these cells do contain small numbers of receptors it is difficult to explain the stimulatory effect without invoking the possibility of additional receptor subtypes with differ-

Table 4. In vivo responses of transplanted solid human small cell lung cancers to Bn/GRP analogues in athymic nude mice

Analogue/tumor	Analogue type*	Percent inhibition of growth†			
		>40	20–40	20	0
NCI-H345 (GRP receptor +)					
D-Cpa-6,ψLeu-13-Phe-14-Bn(6-14)	Antag/Ag		•		
D-Phe-1,Leu-8,9-litorin	Ag		•		
D-Phe-6,ψLeu-13-Cpa-14-Bn(6-14)	Antag	•			
NCI-H69 (GRP receptor −)					
D-Cpa-6,ψLeu-13-Phe-14-Bn(6-14)	Antag/Ag				•
D-Phe-1,Leu-8,9-litorin	Ag			•	
D-Phe-6,ψLeu-13-Cpa-14-Bn(6-14)	Antag				•

*Indicated analogue is either an agonist, antagonist, or mixed antagonist/agonist in rat pancreatic acinar cells (*52*).

†Solid tumors were transplanted subcutaneously into athymic nude mice (eight per group) that were given twice daily sc doses (10–250 μg) of the analogues beginning on day 32 postimplantation and ending on day 84 for H345 and beginning on day 19 and ending day 38 for H69 tumors. Responses shown were the maximum for a given range of doses and were generally achieved at the 50 μg level.

ent activation requirements. In contrast, the pure agonist had little effect in this system.

Overall, the situation with respect to Bn analogue effects on lung tumors is promising but clearly far from simple, and additional work on further analogue and tumor types must be carried out in order to clarify the therapeutic possibilities. Also of great interest and potential will be the effects observed with the various analogues on the in vivo growth of Bn receptor-containing prostate, breast, colon, hepatic, and perhaps other types of tumor.

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Molecular Genetic Analysis of Two Distinct Receptors for Mammalian Bombesin-like Peptides

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ABSTRACT—The mammalian bombesin-like peptides are known to be growth factors for certain cells with high-affinity bombesin receptors and have been implicated as autocrine growth factors influencing the pathogenesis and progression of a subset of human small-cell lung carcinomas. Thus, antagonists that interfere with bombesin receptor-ligand interaction might prove to be of value in treatment of gastrin-releasing peptide (GRP)-responsive tumors. A precise definition of the structure and properties of the bombesin receptors found on human lung cancer cells would provide important information for the design and rational application of such antagonists. Recently, we isolated cDNA clones encoding two distinct receptors for the mammalian bombesin-like peptides, GRP, and neuromedin B (NMB). The two receptors show 56% amino acid identity, encode seven putative transmembrane domains, and are members of the G-protein–coupled receptor superfamily. Ligand-binding studies show that while both receptors can be activated by either GRP or NMB, one receptor has a higher affinity for GRP than for NMB (GRP-R), while the other has a higher affinity for NMB than for GRP (NMB-R). A different spectrum of antagonists is needed to block responses from the two different receptors. These studies indicate that it will be critical in future studies to define which bombesin receptor subtypes are present on a given tumor to optimize the potential therapeutic benefit of antagonists in blocking growth. [J Natl Cancer Inst Monogr 13:141–144, 1992]

Bombesin is a 14 amino acid peptide originally purified from frog skin. Two mammalian bombesin-like peptides with structural and functional similarity to bombesin have been identified and characterized to date. These two peptides are called gastrin-releasing peptide (GRP) and neuromedin B (NMB). The structural similarity shared by bombesin (BN), GRP, and NMB peptides is highest over the seven amino acid residues that comprise the alpha-amidated carboxy termini of the three peptides (BN: Trp-Ala-Val-Gly-His-Leu-Met-NH_2; human GRP: Trp-Ala-Val-Gly-His-Leu-Met-NH_2; human NMB: Trp-Ala-Thr-Gly-His-Phe-Met-NH_2). This conserved domain appears to be necessary and sufficient by itself for high-affinity binding to receptors for BN-like peptides reported in a variety of cell types (*1,2*).

GRP and NMB are potent regulatory peptides of importance in a wide spectrum of cell physiologic processes, including secretion, smooth-muscle contraction, and neuron firing rate (*1,2*). BN and mammalian BN-like peptides can also function as growth factors for Swiss 3T3 fibroblasts (*3*) and have been implicated as autocrine growth factors of importance in some, but not all, human small-cell lung carcinomas (SCLC) (*4*). Most SCLC cells express preproGRP mRNA (*5*), and many SCLC cell lines show stimulation of soft agar colony formation in the presence of either GRP or BN (*6*). A monoclonal antibody directed against the carboxy terminal receptor-binding domain shared by both BN and GRP inhibited the soft agar colony formation of two SCLC cell lines and inhibited the growth of nude mouse xenografts from one SCLC cell line in vivo. The effects of this monoclonal antibody were not observed with other class-matched antisera; inhibition of soft agar colony formation was reversed by addition of 50 n*M* BN in conjunction with the antibody, indicating that the effects observed were specific (*4*).

Given these observations, the BN receptor mediating these effects is a rational target for potent and specific antagonists that might prove useful as therapeutic agents. The low density of receptors often found on responsive SCLC cell lines has confounded a precise definition of the molecular and pharmacologic properties of these receptors in different tumor samples. This issue is particularly relevant in that recent binding studies indicate that there are at least two distinct BN receptor subtypes, with different affinities for GRP and NMB (*7*). Many of the available BN receptor antagonists are effective in blocking responses in only one of the two receptors (*8*).

Recent molecular cloning studies have proven useful in defining precisely the molecular and pharmacologic properties of BN receptor subtypes. Recently, we (*9*) and others (*10*), have isolated and characterized a cDNA clone encoding the murine Swiss 3T3 BN receptor, with higher affinity for GRP than for NMB. Using the Swiss 3T3 receptor cDNA as a low-stringency cDNA probe, we isolated a second distinct BN receptor from rat esophagus. The second receptor has higher affinity for NMB than for GRP (*11*). In this work, the properties of these two receptors

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are summarized, and issues relating to tumor typing and antagonist-mediated growth modulation are discussed.

RESULTS

Fig. 1 shows a comparison of the primary amino acid sequences of the mouse GRP-receptor (GRP-R) (*top*) and rat NMB-receptor (NMB-R) (*bottom*) as determined from nucleotide sequence analysis of cDNA clones isolated from Swiss 3T3 murine fibroblast (GRP-R) (*9*) and rat esophagus (NMB-R) cDNA libraries (*11*). Overall, the two sequences show 54% identity. Hydropathy plots show that there are seven hydrophobic regions in the two proteins (boxed and numbered), indicating that both receptors are members of the rapidly expanding family of seven-transmembrane, G-protein–coupled receptor genes (*12*). This structure predicts that the amino terminal end of the protein will be extracellular, followed by three interdigitated intracellular and extracellular loops and a carboxy terminal intracellular domain.

Several other potentially interesting structural motifs are also noted in this comparison. There are numerous sites of potential *N*-linked glycosylation (Asn-X-Ser/Thr), consistent with earlier reports indicating that the Swiss 3T3 GRP-R is highly glycosylated and that its apparent molecular mass on sodium dodecyl sulfate gels is reduced from 75 kd to about 45 kd by *N*-glycanase treatment (*13, 14*). Two of these potential sites are found in similar regions of both the GRP-R and NMB-R (amino terminal domain; AA 5 GRP-R, AA 8 NMB-R, and second intracellular loop; AA 191 GRP-R, AA 192 NMB-R). Two consensus sites for protein kinase C phosphorylation (*heavy boxes*) (*15*) are also found at conserved locations in intracellular domains of both the GRP-R and NMB-R. No sequence similarities with any of the known tyrosine kinase receptors were found in either the GRP-R or the NMB-R.

Table 1 summarizes the results of whole cell ligand-binding studies performed under conditions described previously (*11,13*) and comparing GRP-R and NMB-R. The relative ligand affinity of the two receptors was assessed by quantitative displacement of [^{125}I-Tyr^4]BN binding by either GRP, NMB, or the specific GRP-R antagonist [D-Phe^6]BN(6-13) ethyl ester (*16*). For analysis of the GRP-R, Swiss 3T3 cells that expressed about 100 000 receptors per cell were used. To compare the binding properties of the two receptors, the NMB-R was expressed at high levels after stable transfection of the cloned rat NMB-R cDNA into mouse fibroblasts that do not normally express BN receptors (Balb 3T3). Both GRP and the GRP-R antagonist are more potent than NMB in competing for labeled BN binding in the Swiss 3T3 GRP-R. In contrast, NMB is more potent than GRP in displacing labeled BN from the NMB-R. Significantly, the affinity of the NMB-R for the GRP-R antagonist is very low. It is clear from these studies that the [D-Phe^6]BN(6-13) ethyl ester compound, a potent antagonist of GRP-R function, is a relatively ineffective antagonist for the NMB-R.

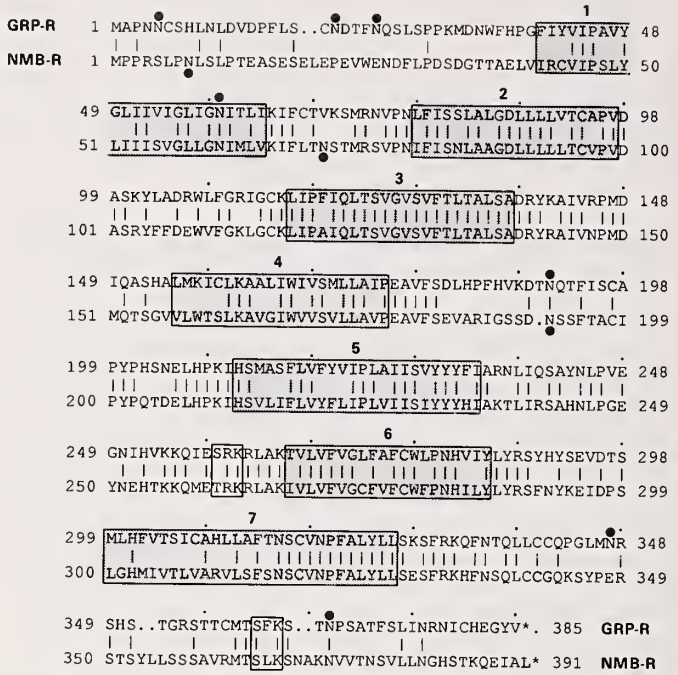

Fig. 1. Comparison of the predicted amino acid sequences of the Swiss 3T3 murine GRP-R and the rat esophagus NMB-R. The primary structure of the GRP-R (top sequence, 384 amino acids) and NMB-R (bottom sequence, 390 amino acids) is compared. *Vertical hatches* designate identical amino acids (overall 54% amino acid identity). *Heavy dots* designate potential sites of *N*-linked glycosylation. *Numbered shaded boxes* show the approximate locations of seven hydrophobic putative transmembrane segments of the two proteins, characteristic of the family of G-protein–coupled receptors. *Open boxes* show the positions of two consensus protein kinase C-phosphorylation sites conserved in both the GRP-R and NMB-R sequence, which may play a role in ligand-dependent receptor desensitization.

Table 1. Displacement of (^{125}I–Tyr^4) BN binding by GRP, NMB, and [D-Phe^6]BN(6-13) ethyl ester antagonist

	Kj, n*M*		
Receptor	NMB	GRP	[D–Phe^6]BN (6-13)ethyl ester antagonist
NMB-R	2	43	>1000
GRP-R	62	2	2

These conclusions are confirmed by studies of ligand-dependent receptor function performed in Xenopus oocytes expressing in vitro transcripts derived from either the cloned Swiss 3T3 GRP-R or rat NMB-R (*9,11*). After injection and expression of an in vitro transcript generated from a cDNA clone template, ligand activation of BN receptors expressed in Xenopus oocytes results in a transient elevation of intracellular calcium, opening a calcium-activated chloride channel. By means of this functional assay system, the ligand-dependent activation of the cloned GRP-R and NMB-R was compared by electrophysiologic measurement of this transient chloride current

in a previous study (*11*). The Swiss 3T3 GRP-R was responsive to 10^{-9}M concentrations of GRP but required 10^{-7}M concentrations of NMB to produce similar responses. In contrast, the rat NMB-R responded to 10^{-9}M concentrations of NMB but required 10^{-7}M concentrations of GRP to produce a response. As expected from the ligand displacement analysis, the [D-Phe^6]BN(6-13) ethyl ester antagonist completely blocked responses of the GRP-R to both ligands when present in a 10-fold molar excess but had no detectable effect on responses from the NMB-R. These studies show clearly that a potent GRP-R antagonist has very different functional and binding properties in GRP-R and NMB-R.

DISCUSSION

Molecular cloning studies have clarified the structure and properties of two distinct receptor subtypes for mammalian BN-like GRP and NMB peptides. The order of ligand affinities differs for the two subtypes; the affinity for a potent GRP-R antagonist is dramatically different in the two receptors. Due to the low numbers of receptors expressed on the cell surface, previous pharmacologic studies characterizing the BN receptors present on human lung cancer cells were not able to distinguish which subtype(s) are expressed and functional. Given the differences in antagonist sensitivity, it will be of great importance in future studies designed to modulate BN-dependent growth of SCLC to determine the subtype composition of a given tumor before embarking on any potential therapeutic intervention. It is also possible that a third BN receptor subtype structurally and pharmacologically distinct from either receptor characterized to date may also be important. Relaxed stringency molecular cloning studies to isolate cDNA clones for the human counterparts to the Swiss 3T3 GRP-R, the rat NMB-R, and any other structurally related cDNAs will be important in establishing which receptors are expressed by BN-responsive human lung cancer cells.

Both GRP-R and NMB-R share structural similarity with other members of the G-protein–coupled receptor gene superfamily, including seven hydrophobic regions believed to be transmembrane segments. The Swiss 3T3 GRP-R is one of the first members of this family shown to be a growth factor receptor. The growth-promoting potential of the NMB-R remains to be clearly demonstrated, although preliminary studies show that Balb 3T3 fibroblasts expressing a transfected NMB-R gene show ligand-dependent activation of second messenger pathways including elevation of intracellular Ca^{++} and phosphatidyl inositol turnover (Sausville E: personal communication). It will be of interest in future studies to determine if G-protein–coupled, calcium-mobilizing receptors for other peptide ligands can also regulate growth in certain cellular contexts.

Both GRP-R and NMB-R become refractory to repeated applications of agonists, suggesting the possibility that there may be ligand-activated, post-translational changes in the protein that desensitize these receptors to further agonist stimulation. Zachary and co-workers (*17*) have shown that the early cellular responses following stimulation of the Swiss 3T3 GRP-R include activation of protein kinase C. In SCLC cells, pretreatment with phorbol esters attenuated BN-stimulated increases in intracellular calcium. Taken together, these observations suggest that protein kinase C might be important in mediating BN receptor desensitization, possibly by phosphorylating sites on the receptor protein itself. Notably, two consensus protein kinase C phosphorylation sites (*15*) are found at identical locations in the third intracellular loop and the carboxy terminal intracellular domain in both GRP-R and NMB-R sequences. Future studies using site-directed mutagenesis will be needed to determine whether or not these sites are important in regulating receptor responses to agonists.

Autocrine growth stimulation of SCLC cells by BN-like peptides is but one of many molecular mechanisms shown to be important in the pathogenesis and progression of lung cancer. In addition to growth factor stimulation, activation of dominant proto-oncogenes and deletion or mutation of tumor suppressor genes are critical somatic genetic changes that appear to promote tumor cell growth (*18*). Human lung cancer cells typically show evidence of multiple genetic changes. Given this complex milieu of genetic changes, the sum of which results in growth deregulation, it is not clear in what percentage of cases antagonist-mediated blockades of BN receptor function will substantively affect the growth of lung tumor cells in the clinical setting. It should be noted, however, that the BN receptor is a pharmacologically accessible target on the cell surface, in contrast to many of the nuclear regulatory proteins encoded by proto-oncogenes and tumor suppressor genes. With a more precise definition of the structural and pharmacologic properties of BN receptors expressed in a given human tumor, it is hoped that it will be possible in the future to predict which tumors are likely to be sensitive to specific BN receptor antagonists and to develop better antagonists for subtypes that are not responsive to currently available compounds.

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Effects of Neuropeptides on Human Lung and Breast Cancer Cells[1]

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ABSTRACT—We evaluated the effect of seven classes of neuropeptides [bradykinin, cholecystokinin 26-33 (CCK), neurotensin, arginine-8 vasopressin (AVP), tyr-4 bombesin (BN), somatostatin, and motilin] on 18 human lung cancer and four human breast cancer cell lines to determine the pattern of responses. Flow cytometric analysis of Indo-1 AM-loaded cells was used to quantitate the intracellular calcium response of individual cells produced by these peptides alone or in simultaneous or sequential combinations. All 18 lung cancer cell lines responded to one or more peptide classes with classic small cell lines displaying the greatest responsiveness, followed by variant small-cell lines and non-small-cell lung cancer cell lines. Breast cancer cell lines demonstrated little or no response to any peptide. There was great variability in the magnitude of response and pattern of response in individual cell lines and between cell lines. Bradykinin was the most potent peptide and produced responses in the largest number of lung cancer cell lines. Simultaneous administration of active peptides produced greater intracellular calcium release than single peptides, though in a less than additive manner. Response to each peptide was followed by a refractory period lasting several hours or more. The refractoriness was peptide-specific, implying that each peptide has a distinct pathway, at least at the receptor level. Bradykinin antagonists could abrogate the calcium response to bradykinin but not to other peptides. Similarly, specific peptide antagonists for CCK, BN, and AVP blocked the response for only their specific agonist. The Substance P-derivative [D-Arg^{1}, D-Phe^{5}, D-Trp^{7,9}, Leu^{11}]-Substance P completely inhibited the responses to BN, AVP, and bradykinin and partially to CCK. The clinical antitumor utility of these antagonists alone or in combination with other neuropeptide antagonists is under investigation. [J Natl Cancer Inst Monogr 13:145-151, 1992]

Shortly after the mammalian peptide bombesin was discovered, it was reported to be present in the majority of small-cell lung cancers (SCLC) (*1,2*). The human analogue of bombesin was shown to be gastrin-releasing peptide. Subsequent studies demonstrated that some SCLC cell lines produced and secreted bombesin, increased their growth in the presence of nanomolar concentrations of bombesin, had specific bombesin receptors, and demonstrated growth inhibition in the presence of anti-bombesin antibodies (*3–5*). Bombesin was thus felt to be an autocrine growth factor for SCLC and great enthusiasm was generated for developing new therapeutic approaches by interfering with this pathway.

The intracellular signal pathway used by bombesin was shown to involve coupling of the receptor to a G-protein, which led to activation of phospholipase C (PLC) (*6,7*). This membrane enzyme cleaves membrane lipids to inositol trisphosphate, which in turn releases calcium from stores in the endoplasmic reticulum. The free intracellular calcium binds to calmodulin and other proteins. The activated PLC also leads to activation of diacylglycerol and then to protein kinase C (*8*). Through unknown pathways, the activation of protein kinase C and the rise in intracellular calcium lead to nuclear signals that lead to activation of c-myc and fos and cell proliferation (*9,10*).

Bombesin antagonists were synthesized and were shown to block the intracellular signals induced by bombesin (*11–13*). In some systems, growth inhibition was shown by these compounds as well. However, other recent studies indicated that solely blocking the effects of bombesin might fail to produce anti-tumor effects in most SCLC patients. These data include studies indicating that 1) many cell lines fail to respond to bombesin (*12,14*); 2) there are many other growth factors for lung cancer cells, including other neuropeptides (*14,15*); 3) anti-bombesin antibodies given in vivo require long-term administration, work best in preclinical tumors, and fail against some cell lines (*16*); and 4) bombesin antagonists have inconsistent anti-tumor effects (*12*).

The endocrine features of SCLC, including the production of a variety of peptide hormones, have been recognized for many years. Many of these peptide hormones have been reported to have growth stimulatory effects on various types of malignancies. In this report we conducted a series of studies evaluating several neuropeptides and peptide antagonists using an automated fluorescence assay of intracellular calcium levels to assess response in lung cancer cell lines with breast cancer cell lines used as controls.

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MATERIALS AND METHODS

Cell Lines

Human SCLC cell lines NCI-H69, NCI-H146, NCI-H209, NCI-H345, NCI-H446, NCI-H510, NCI-H526, NCI-N417, and human non-small-cell lung cancer (NSCLC) cell lines NCI-H125, NCI-H460, and A549 were obtained from Drs. J. Minna, A. Gazdar, and D. Carney at the National Cancer Institute, Bethesda, Md., or from the American Type Culture Collection, Rockville, Md. The SCLC cell line SHP-77 was obtained from Dr. A. Koros, University of Pittsburgh, Pittsburgh, Pa. The SCLC cell lines DMS153 and DMS114 were obtained from Dr. O. Pettingill, Dartmouth Medical School, Hanover, N.H., and UMC1A and UMC19 were obtained from Dr. B. Poiesz, State University of New York Upstate, Syracuse, N.Y. Human NSCLC cell line UCLC-11 was established in our own laboratory, and Colo-699 was obtained from Dr. G. Moore, University of Colorado Cancer Center, Denver, Colo. The human breast cancer cell lines ZR-75, MCF-7, T470, and MDA-231 were obtained from Dr. K. Horwitz, University of Colorado Cancer Center, Denver, Colo. Cells were grown in 5% CO_2 incubators at 37 °C with 100% humidity. They were maintained in serum-free medium containing hydrocortisone, insulin, transferrin, estradiol, and selenium (HITES) (*17*), ACL-3 medium (*18*), or 10% fetal calf serum.

Peptides

Tyr-4 bombesin, bradykinin, cholecystokinin 26-33, gastrin-I, arginine-8 vasopressin, neurotensin, motilin, somatostatin, Substance P, and [D-Arg^1, D-Phe^5, D-Try^{7,9}, Leu^{11}]-Substance P were obtained from Peninsula Labs, Belmont, Calif. The calcium ionophore ionomycin was obtained from Calbiochem, La Jolla, Calif.

Flow Cytometry

Flow cytometric analyses were performed with an EPICS 752 cell sorter (Coulter Electronics, Hialeah, Fla.) using 409-nm and 490-band pass filters (Oriel Corp., Stratford, Conn.) and an Innova 90/5 argon ion laser (Coherent, Palo Alto, Calif.). Forward angle and 90° light scatter from 490-nm fluorescence was used for gating viably loaded cells. The ratio of 410-nm:490-nm fluorescence emission was calculated digitally on a cell-by-cell basis using the MDADS hardware (Coulter Electronics). The 410- and 490-nm emission fluorescence and the 410:490 ratio were collected versus time. Instrument software was used to display ratio fluorescence versus time and the percent of cells responding versus time. Analysis of individual histogram time slices was done on Coulter EASY88 computer from 64 × 64 dual parameter histograms.

Intracellular Calcium Assays

Intracellular calcium levels were assayed using the fluorescent probe Indo-1AM (Molecular Probes, Eugene, Ore.). Single-cell suspensions (2.5×10^6 cells/mL) were suspended in 20 n*M* HEPES/BSS buffer adjusted to pH 6.8 at 37 °C, as previously described (*14*). We incubated 5 μM Indo-1AM with the cells for 30 minutes at 37 °C, then diluted 1:1 with 20 n*M* HEPES/BSS buffer adjusted to pH 7.4 to raise the final pH to 7.1, followed by a second 30 minutes of incubation at 37 °C. The cells were washed twice in HEPES/BSS buffer (pH 7.4 at 37 °C) containing dextrose and resuspended to 1×10^6 cells/mL. Indo-1-loaded cells were analyzed for their intracellular calcium level in the EPICS 752 cell sorter. After resting levels were obtained, specific peptides were added. This caused a short (10-second) interruption in the analysis. Ionomycin (4 μM) was used as a stimulus after each experiment to ensure that adequate Indo-1AM loading had occurred.

[^{3}H] Thymidine

Cells were plated in serum-free media at a concentration of 5000 live cells per well in 96-well plates (Corning 25860). HITES medium was used for SCLC. ACL-3 medium was used for all other lines studied. Cells were allowed to settle for 4 hours at 37 °C in a 5% CO_2 incubator before applying peptide agonists or antagonists. Following a 24-hour incubation at 37 °C and 5% CO_2, 0.4 μCi [methyl-^{3}H] thymidine (ICN Biomedicals) was added in a volume of 10 μL RPMI medium. Cells were incubated for an additional 24 hours in a 37 °C 5% CO_2 incubator before harvesting cells using a Titertek cell harvester. One-minute sample counts were done using a Beckman LS1801 scintillation counter (Beckman Scientific). Counts were performed in sets of eight, and mean values were compared to control values of counts in media without peptide agonist or antagonist addition.

RESULTS

Effects of Individual Peptides

The intracellular calcium level (ratio 410:490) rose rapidly after the addition of peptide stimuli such as cholecystokinin 26-33 (Fig. 1A). Maximal response occurred within 30 seconds. The $[Ca^{2+}]i$ levels returned to baseline within 3 minutes. The analysis of individual cells by flow cytometry allowed for calculation of the fraction of cells responding. Fig. 1B shows the percentage of cells with an increase in $[Ca^{2+}]i$ over time. A maximum of 70% of the NCI-H510 SCLC cells responded to the cholecystokinin 26-33.

All of the neuropeptides studied were quite potent, with maximal responses observed between 1 n*M* and 100 n*M*. The dose response of cholecystokinin 26-33 on NCI-H510 cells is shown in Fig. 2. A dose of 1 n*M* produced some response, and a peak response was obtained with 100 n*M*. The response to 1000 n*M* was identical to that produced by 100 n*M*. For most of the peptides evaluated, a concentration of 50–100 n*M* produced maximal effects on lung cancer cell lines. One exception to this was the effect of

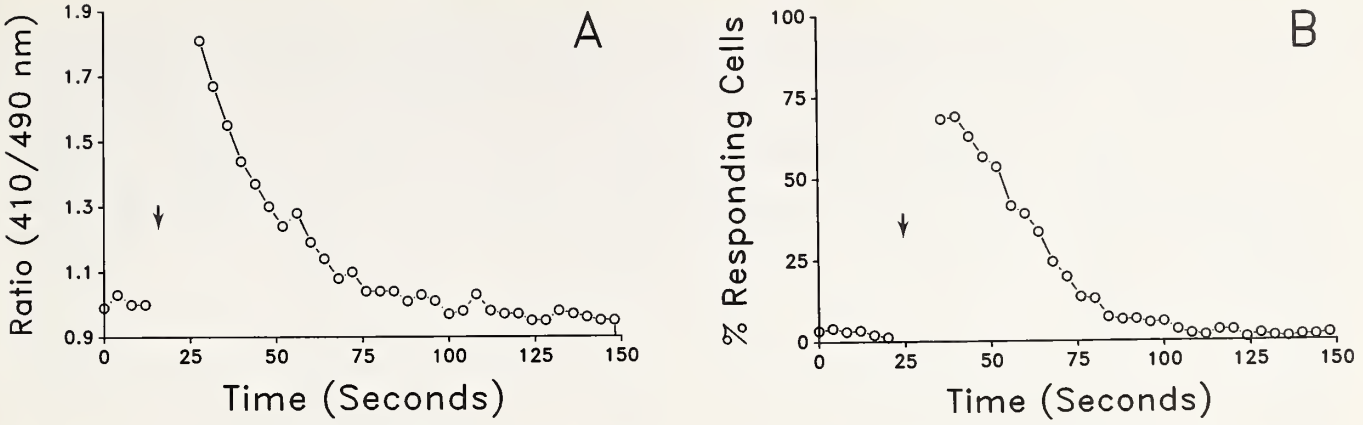

Fig. 1. Response of NCI-H510 "classic" SCLC cells to 100 n*M* cholecystokinin 26-33. Fig. 1A shows the 410/490 nm ratio (intracellular calcium) versus time. The cholecystokinin was applied at 15 seconds, and within 15 seconds the intracellular calcium level rose to peak levels, and returned to baseline by 2 minutes. Fig. 1B shows the fraction of cells responding. The majority of cells (70%) responded with an increase in intracellular calcium, but 30% of cells had no response.

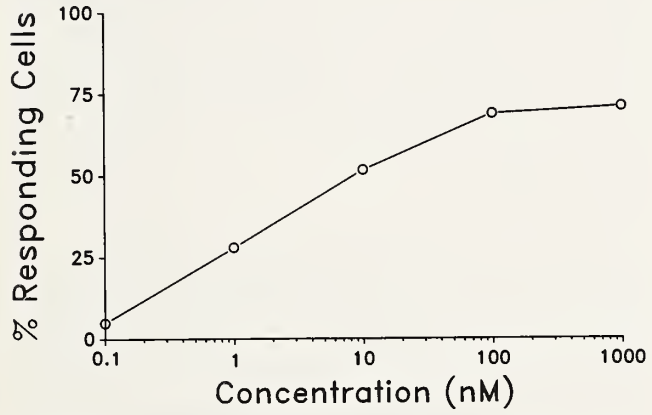

Fig. 2. Dose-response effects of cholecystokinin 26-33 on NCI-H510 "classic" SCLC cells. There was no response to 0.1 n*M*. Concentrations of 1.0 and 10 n*M* produced intermediate responses, and concentrations of 100 and 1000 n*M* produced maximal responses.

bradykinin on SHP-77 cells where 1-10 n*M* produced maximal effects.

Each peptide evaluated (cholecystokinin 26-33, gastrin-I, bradykinin, tyr-4 bombesin, arginine-8 vasopressin, somatostatin, and motilin) produced a calcium response in some but not all small cell lung cancer cell lines (Table 1). Cholecystokinin 26-33 and neurotensin produced responses in all seven "classic" small-cell lines but only in two of six "variant" small-cell lines. Bradykinin, tyr-4 bombesin, and arginine-8 vasopressin produced a response in six out of seven "classic" SCLC cell lines and in four, two, and one "variant" SCLC cell lines, respectively. Motilin and somatostatin each produced responses in eight of the 13 SCLC cell lines. Overall, responses were more frequently observed in "classic" than in "variant" SCLC cell lines (84% versus 43%).

Bradykinin produced responses most frequently in the NSCLC cell lines (five out of five) (Table 1). Neurotensin, somatostatin, and motilin produced responses in three out of five NSCLC cell lines, whereas cholecystokinin, bombesin, and arginine-8 vasopressin each produced responses in two out of five NSCLC cell lines.

When all lung cancer cell lines are considered, bradykinin produced a response in 15 of 18 cell lines; followed by neurotensin (12 of 18); cholecystokinin, motilin, somatostatin (11 of 18 each); tyr-4 bombesin (10 of 18); and arginine-8 vasopressin (9 of 18).

The rises in intracellular calcium were produced by release from intracellular stores, not by changes in membrane calcium channels. There were no differences in peptide effects when the peptide was added in calcium-free medium with or without EGTA. High concentrations (4 μM) of ionomycin produced prolonged rises in intracellular calcium. It is not clear whether this was the result of toxic effects or of membrane calcium channels.

Peptide Combinations

Each of these peptides used separate and distinct signal pathways. It is uncertain whether the differences are solely at the receptor level, whether they are coupled to distinct G-proteins, and whether subsequent signal events are distinct or identical. Combinations of various peptides produced sub-additive results. For example, in NCI-H510 cells cholecystokinin 26-33 produced a response in 70% of the cells, bradykinin in 17%, and bombesin in 19%. The combination of bradykinin and bombesin produced a response in 25% of the cells, and the combination of the three peptides produced a response in 76% of the cells. This same three-peptide combination produced a response in 80% of SHP-77 cells, 75% of UCLC-11 cells, and 69% of NCI-H345 cells. The addition of more peptides resulted in only a small percentage of increase in the number of cells responding.

Pathway/receptor down-regulation occurred after each peptide stimulus, and this was peptide specific. When intracellular calcium levels returned to baseline, 2 to 3 minutes after the addition of a peptide stimulus, the sub-

Table 1. Response of lung and breast cancer cell lines to seven different neuropeptides

Peptide	Classic N = 7			Variant N = 6			NSCLC N = 5			Breast N = 4		
	No. of cell lines responding	% of cells responding		No. of cell lines responding	% of cells responding		No. of cell lines responding	% of cells responding		No. of cell lines responding	% of cells responding	
		Mean	Range		Mean	Range		Mean	Range		Mean	Range
Cholecystokinin	7	43	12–81	2	11.5	11–12	2	13.5	13–14	0	0	0
Bradykinin	6	18	11–28	4	43	13–83	5	34	13–61	0	0	0
Tyr-4 bombesin	6	19.5	11–45	2	11.5	11–12	2	38.5	23–54	0	0	0
Arginine-8 vasopressin	6	27	10–54	1	27	27	2	12	11–13	1	14	14
Neurotensin	7	24	10–43	2	12	12	3	28	18–42	0	0	0
Motilin	5	14	10–18	3	11	10–12	3	19	11–27	1	13	13
Somatostatin	4	13	10–16	4	15	10–24	3	25	10–36	0	0	0
Total	41 of 49 84%			18 of 42 43%			20 of 35 57%			2 of 28 7%		

sequent administration of the same peptide produced no response. For example, the sequential administration of 100 n*M* cholecystokinin 26-33 or the related peptide gastrin I to NCI-H345 cells produced a response only to the first administration (Fig. 3A). At the time the cells were refractory to cholecystokinin 26-33 or gastrin I, however, they were fully responsive to other peptides such as bradykinin or bombesin (Fig. 3B).

Effects of Neuropeptides on Cell Proliferation

There was no direct correlation between peptide effects on intracellular calcium and cell proliferation. For example, 83% of SHP-77 cells exhibit increases in $[Ca^{2+}]i$ in response to bradykinin. However, the same and higher concentrations of bradykinin had no effect on [^{3}H]thymidine incorporation on SHP-77 cells (Fig. 4). A lack of effect on cell proliferation was observed in most instances when single peptides were added to these lung cancer cell lines. It is not clear whether multiple peptides or peptides in combination with other growth factors are required to induce proliferation.

Effects of Peptide Antagonists

We examined a number of peptide antagonists for their ability to inhibit signal transduction. The Substance P derivative [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}]-Substance P blocked the calcium response to bombesin, bradykinin, and arginine-8 vasopressin but had only a partial inhibitory effect on the calcium response induced by cholecystokinin 26-33 (Fig. 5). The concentration of the antagonist to produce a 50% inhibition (IC_{50}) of 100 n*M* of the stimulating peptide was 1 μM with complete inhibition at 5 μM. Peptide antagonists that were more potent were specific for a single peptide.

We next examined the effects of this substance P derivative on [^{3}H]thymidine incorporation in SCLC cell lines (Fig. 6). There was no effect of 1-μM concentration on [^{3}H]thymidine incorporation. A small inhibitory effect was seen at 10 μM, and the IC_{50} was 20 μM. A concentration of 40 μM was required for complete inhibition. These concentrations were much higher than the concentrations required for inhibiting calcium response. The effects could not be reversed by the addition of tyr-4 bombesin (data not shown). More potent but peptide-specific antagonists had no effect on proliferation.

DISCUSSION

Multiple neuropeptides induced intracellular signal events in lung cancer cell lines. The signal pathway includes activation of phospholipase C with cleavage of membrane phosphoinosotides to inositol trisphosphate and release of calcium from intracellular stores (*6–8*). These signals are transmitted within 10 seconds of peptide exposure, and cells return to their baseline calcium levels within minutes. However, the cells remain refractory to subsequent administration of the same peptide for more than 6 hours. The response and down-regulation are peptide specific, as cells may respond to sequential administration of peptides with different amino acid sequences and receptors.

The signal pathways induced in lung cancer cells by these neuropeptides were present in all lung cancer cell lines examined but were not present in four breast cancer cell lines. SCLC cell lines were more responsive than NSCLC cell lines. Within SCLC cell lines, the "classic" lines were more responsive than "variant" lines. These features are consistent with the hypothesis that all lung cancers are derived from a common stem cell and that non-small-cell lung cancers often express neuroendocrine features (*19,20*).

There was great heterogeneity of response to various neuropeptides both within individual lung cancer cell lines and between lung cancer cell lines. In most instances a minority of cells within a cell line responded to an individual peptide. It is not clear whether this is due to a lack of expression of the receptor for these peptides in the nonresponsive cells or whether there is autocrine secretion of peptide and some cells are self down-regulated at the time

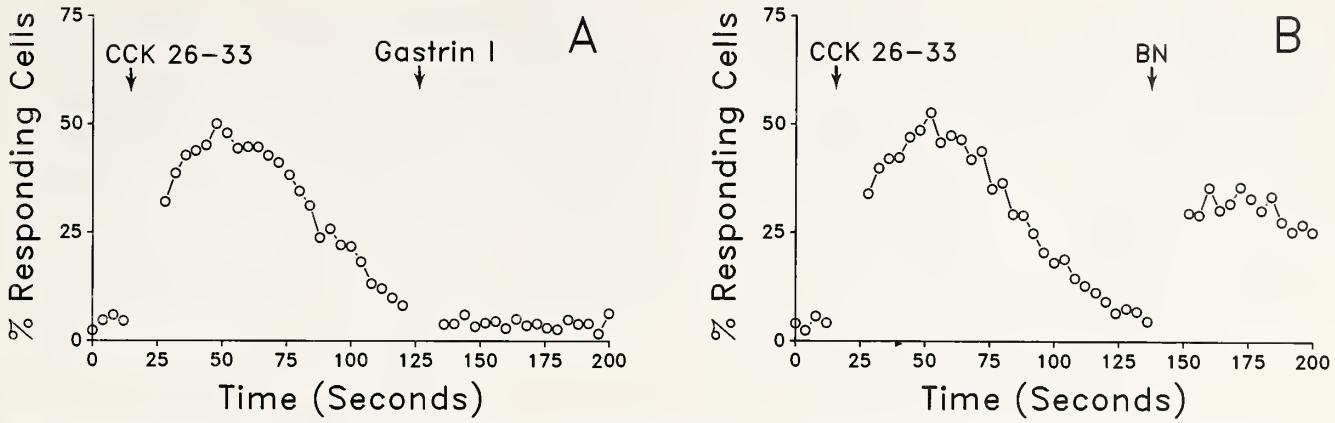

Fig. 3. Response of NCI-H345 "classic" SCLC cells to the sequential administration of peptides. In Fig. 3A cholecystokinin 26-33 (100 n*M*) was added after 15 seconds. When the cells had returned to baseline intracellular calcium levels after 2 minutes, 100 n*M* gastrin-I was added, and there was no subsequent response. Similarly, 100 n*M* cholecystokinin 26-33 produced no response at the second application. In Fig. 3B 100 n*M* cholecystokinin was added after 20 seconds. When the intracellular calcium levels returned to baseline levels, 100 n*M* tyr-4 bombesin was added, and the typical response to tyr-4 bombesin ensued.

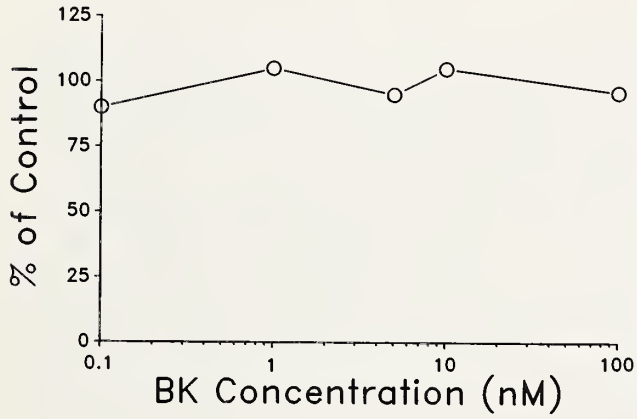

Fig. 4. Proliferative response of SHP-77 SCLC cells to 100 n*M* bradykinin. The incorporation of [^{3}H] thymidine in SHP-77 cells incubated with 100 n*M* bradykinin was identical to the [^{3}H] thymidine incorporation of SHP-77 cells grown in RPMI medium alone over a broad concentration of bradykinin. In calcium flux assays, 1 n*M* bradykinin produced a response in 83% of SHP-77 cells.

of peptide administration. It is possible that nonresponding cells could be sorted on a fluorescence-activated cell sorter and followed over time. If a proportion of these cells subsequently become responsive, it would suggest that autocrine production may have down-regulated the cells. If the cells remained unresponsive, it would suggest that the cells lacked receptor expression.

The sub-additive effects of peptide combinations confirmed the marked heterogeneity of receptor expression. Some cell lines express receptors for only one peptide, some express receptors for two or more peptides, and some cell lines do not express receptors for any of the peptides. In general, combinations of three peptides produced a response in 70% to 80% of cells. The addition of more peptides failed to produce a response in 100% of cells. However, all cells were capable of response as documented by response to ionomycin.

These data have profound implications for attempts to develop new treatment and prevention strategies. The studies of Aguayo et al (*21*) and Willey et al (*22*) showed bombesin is mitogenic for pulmonary fibroblasts and bronchial epithelial cells. Smokers were shown to have higher levels of bombesin in their bronchial lavage fluid than nonsmokers. It has been postulated that pulmonary injury produced by carcinogens in tobacco smoke induces production and release of these neuropeptides. These, in turn, produce proliferation and hyperplasia of bronchial epithelial cells. Subsequent events cause malignant transformation. The malignant cells are often dependent on the same growth factors. However, during tumor progression the cells may become independent of autocrine growth factor stimulation.

The heterogeneity of neuropeptide responsiveness must be considered in developing new treatment and prevention strategies. Antibodies to single growth factors or growth factor receptors may be poor candidates for treatment or prevention. They would require continuous exposure and would affect only a minority of cells. Antireceptor antibodies conjugated to penetrating radioisotopes, such as ^{131}I or ^{90}Y, could be effective since neighboring cells without receptors might be killed along with receptor positive cells.

Highly specific and highly potent peptide antagonists would be predicted to produce growth inhibition in a minority of instances. These antagonists may be similarly conjugated to penetrating radioisotopes to kill receptor negative as well as receptor positive cells. If unlabeled antagonists were used, near continuous exposure would be required as the agents would be cytostatic, not cytotoxic. In addition, multiple antagonists would be required, which would be costly and potentially toxic. The pharmacokinetics of serum disappearance would be critical in these instances. Radiolabeled antagonists would be

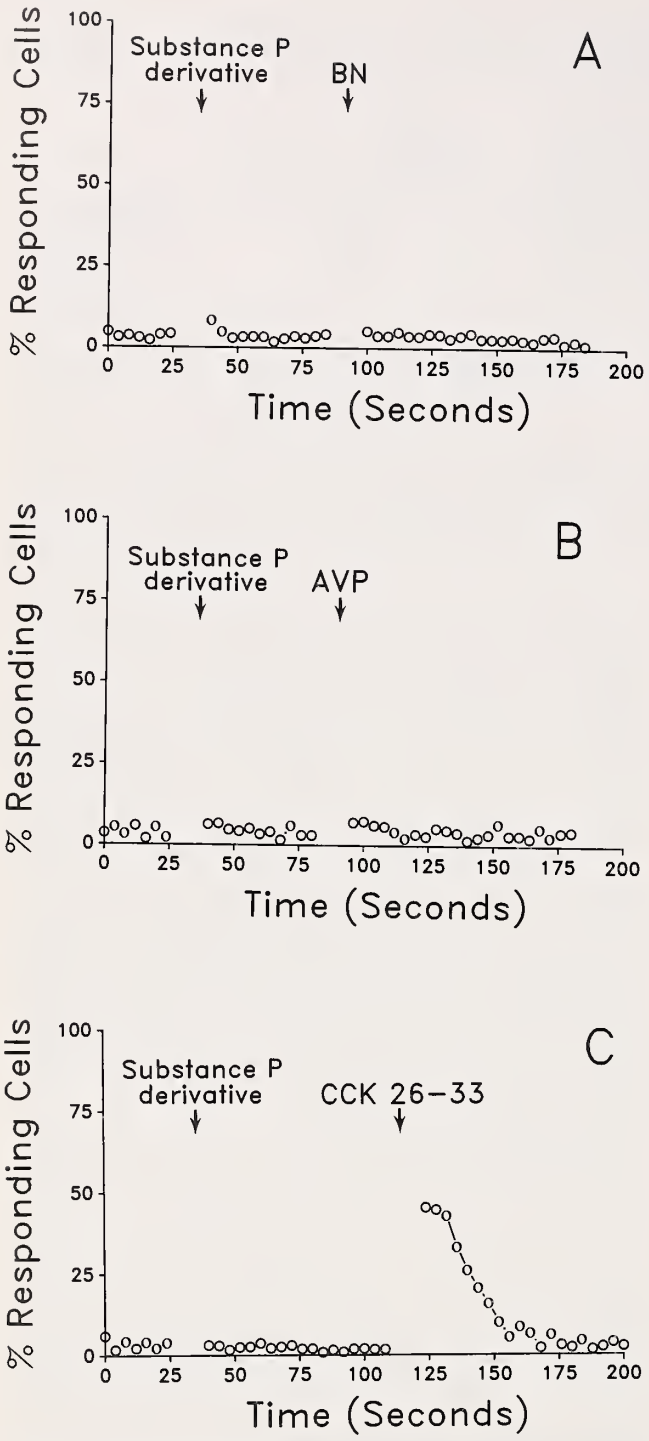

Fig. 5. Calcium response of SCLC cells to the sequential administration of 5 μM [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}]-Substance P followed by application of 100 nM peptide agonist. In Fig. 5A, 5B, and 5C, 5 μM [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}]-Substance P was added at about 25 seconds and produced no change in intracellular calcium levels. A second peptide was added after 90 to 110 seconds. In Fig. 5A the response of NCI-H345 cells to 100 nM tyr-4 bombesin was entirely blocked. In Fig. 5B the response of NCI-H345 cells to 100 nM arginine-8 vasopressin was entirely blocked. In Fig. 5C the response of NCI-H510 cells to 100 nM cholecystokinin 26-33 was decreased by about 33% (compare Fig. 1B with a response in 70% of cells to Fig. 5B with a response in 45% of cells).

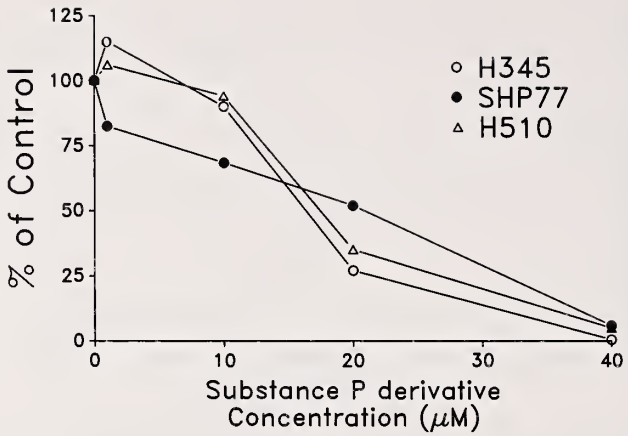

Fig. 6. Inhibition of proliferation of SCLC cells by [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}]-Substance P. The incorporation of [^{3}H] thymidine in the SCLC cell lines NCI-H345, NCI-H510, and SHP-77 was inhibited by increasing concentrations of [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}]-Substance P.

cytotoxic and would not require continuous exposure. They would require careful estimates of biodistribution, however, because receptor positive normal cells would bind the toxic antagonists as well.

It is possible that antagonists could be developed that would bind to multiple neuropeptide receptors. There are a number of specific substitutions in the amino acid sequence that change agonists to antagonists for these neuropeptides. This suggests that specific conformational changes have similarities between receptors. The Substance P derivatives block the effects of many, but not all, neuropeptides. These compounds have been shown to have antitumor effects in vitro and in vivo (*12,13*). These effects require high concentrations, however. It is also not certain that mere blocking of peptide binding produces the antitumor effects. The pharmacokinetics and bio distribution of these compounds in vivo have not been described. These results provide hope that cross-reacting peptide antagonists may be developed that will be stable in vivo.

These growth factor pathways may be blocked at other sites as well. The autocrine production of these neuropeptides requires the PHM (peptidylglycine α-hydoxylating monoxygenase) and PAL (peptidyl α-hydroxyglycine α-amidating lyase) enzymes, as discussed elsewhere (*23,24*). Blocking of these enzymes could inhibit production of the peptides.

The intracellular signal events that occur after ligand receptor binding are similar for the various peptides. Numerous steps in this signal pathway would be amenable to pharmacologic intervention. For example, it is possible to interfere with G-proteins, to block the effects of phospholipase C, to interfere with calcium binding to effector proteins such as calmodulin and block protein-DNA interactions that occur in the nucleus such as jun and fos DNA-protein binding. Clearly, the therapeutic ratio of

antitumor effects versus normal tissue toxicity needs to be determined for each of these possible interventions.

In conclusion, a number of neuropeptides induces intracellular signal events in lung cancer but not in breast cancer cells. The heterogeneity of peptide responsiveness suggests that interference with a single peptide-receptor interaction would have little utility. Nonetheless, there are numerous ways to produce antitumor and antiproliferative effects. Future studies should address the therapeutic and preventive potential of these interventions.

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Differential Stimulation of the Growth of Lung-Metastasizing Tumor Cells by Lung (Paracrine) Growth Factors: Identification of Transferrin-Like Mitogens in Lung Tissue-Conditioned Medium[1]

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ABSTRACT—Certain metastatic tumor cells successfully form metastases at particular organ sites, and their organ colonization properties cannot be explained by mechanical or anatomic factors. These tumor cells possess the ability to colonize such sites through preferential adhesion to organ microvessel endothelial cells, preferential organ invasion by expression of particular degradative enzymes and response to organ motility factors, and preferential organ growth by response to growth factors present at relatively higher concentrations in the target organ. The likelihood that target organ-associated growth factors exist and are important in metastatic colonization has been approached by studying the mitogenic effects of target organ extracts, fragments, or conditioned media on poorly and highly metastatic tumor cells that show organ preference of metastasis. We previously described the isolation of a major organ-derived (paracrine) growth factor from lung tissue-conditioned medium. Characterization of this mitogen has demonstrated that it is a transferrin or a transferrin-like glycoprotein, and antibodies to transferrin can remove significant growth activity from lung tissue-conditioned medium. Further demonstration of the existence and characterization of metastasis-associated organ (paracrine) growth factors and their receptors will be helpful in understanding the organ preference of metastasis. [J Natl Cancer Inst Monogr 13:153–161, 1992]

Paget (*1*) was the first to suggest that certain cancers nonrandomly spread to particular secondary sites on the basis of unique properties. In these cancers, the metastatic colonization of particular sites cannot be explained by mechanical considerations, such as the circulatory linkage of the secondary site to the primary tumor or the configuration of the microcirculatory system. It is well known that mechanical and anatomic considerations, such as mechanical lodgement in the first capillary bed encountered, are important factors in blood-borne tumor spread. But after observing the unique patterns of colonization of breast cancers and microorganisms, Paget (*1*) proposed that metastatic development was not based solely on mechanical and anatomic considerations but was a consequence of particular tumor cells ("seeds") finding a suitable environment ("soil") in order to develop and grow.

We and others have identified some of the tumor cell (seed) properties associated with the organ preference of metastasis (*2–6*). These include their abilities to 1) adhere to the microvascular endothelial cells of the target organ (*5,7–9*); 2) respond to chemotactic signals emanating from the target organ (*10,11*); 3) attach to, invade, and degrade the subendothelial matrix of the target organ (*12,13*); 4) evade the immune surveillance systems of the target organ (*4,14,15*); 5) respond to local growth signals in the target organ (*16–19*) and other properties important in metastasis (*2–6*).

For metastatic colonization to be successful, the appropriate "soil" or organ site must possess several characteristics. These include the correct type of microvessel endothelial cells (*20,21*), organ matrix, or stroma (*8,22*); the presence of paracrine motility factors (*10,11*) and paracrine growth factors (*16–19*); and possibly the interaction of the tumor cells with the appropriate organ effector cells (*23,24*).

To study the organ preference of metastasis, animal and human tumor lines have been selected in vivo or cloned in vitro to produce tumor cell sublines that have an enhanced ability to metastasize to certain organ sites (*4,5,25*). These tumor models have been a valuable tool in studying the phenomenon of site-specific tumor spread and the tumor cell characteristics involved in metastasis formation.

One of the most important characteristics of metastatic cells that might explain their organ preference of metastasis is their ability to preferentially respond to growth factors produced by or situated in the preferred organ for metastasis (*16–19*). To study the ability of organ-preferring tumor cells to respond to growth factors from various organs, in vitro growth responses have been measured using various target and nontarget organ-derived factors prepared from extracts of organs, culture medium conditioned by organs, fragments of organ tissue in culture, or viable cells cultured from particular organs. We have concentrated on experiments that identify organ (paracrine) growth factors that differentially stimulate the growth of highly metastatic cells, and we have begun to isolate and purify such factors (*26,27*).

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MATERIALS AND METHODS

Cell Lines

Murine B16 melanoma sublines of low (B16-F1) and high (B16-F10) lung colonization potentials were obtained from Dr. I.J. Fidler (M.D. Anderson Cancer Center, Houston, Tex.). Rat mammary adenocarcinoma cell lines and clones were derived from the 13762NF tumor (*28*). Line MTPa and clones MTF7 and MTC.4 were derived from the tumor growing at mammary fat pad sites, whereas clones MTLn2 and MTLn3 were derived from lung metastases. Lines PaB5 and PaB10 were independently selected from MTPa five and ten times, respectively, for brain colonization (*29*). All cell lines were grown in vitro in 12-well Corning (Corning, N.Y.) tissue culture plates in a 1:1 mixture of Dulbecco's modified Eagle medium and F12 (DME/F12) medium containing 5% fetal bovine serum (FBS) without antibiotics at 37 °C in a humidified incubator (5% CO_2; 95% air). All cell lines were examined for and found free of *Mycoplasma* contamination using Hoechst 33258 staining and Gene-Probe™ analysis (*30*).

Purification of Lung-Derived Growth Factor-1

Lung-derived growth factor-1 (LDGF-1) was purified from rat lung tissue-conditioned medium as previously described (*26,27*). Briefly, this involved harvesting rat lung tissue-conditioned medium; concentrating the medium using a pressurized Amicon device (YM-10 filter); using dialysis; mixing with hydroxylapatite and preparing a column; eluting with a 10–50 m*M* potassium phosphate gradient; applying active fractions onto a DEAE-Sepharose column and eluting with 0.25 m*M* Tris buffer (pH 8.5); chromatofocusing active fractions using a Polybuffer Exchanger 94 column; applying gel filtration of the active fractions on a Bio-Gel P-200 column; and separating the active fractions by preparative native gel electrophoresis. The final product yielded one silver-stained band of $M_r \sim 66\,000$ on nonreducing or one silver-stained band of $M_r \sim 72\,000$ on reducing SDS-polyacrylamide gel electrophoresis (*26,27*).

Effects of Paracrine Growth Factors on Cell Growth

Cell growth studies and the effects of organ-derived growth factors were performed in tissue culture as described previously using a crystal violet cell quantitative assay (*27*).

Binding of ^{125}I-Labeled Transferrin

Murine or human transferrin (Tf) at 5 mg/mL in phosphate-buffered saline (PBS) was saturated with iron by incubation with ferric ammonium citrate (0.1 mg/mL) in 0.01 *M* $NaHCO_3$ for 4 hours at room temperature. Unbound iron ions were removed by dialysis against PBS overnight. Iron-saturated Tf was ^{125}I-labeled by a modification of the procedures of MacConnhey Rey and Dixon (*31*). Tf (200 μg) was dissolved in 0.5 mL PBS, 0.5 mCi $Na^{125}I$ and 10 mL chloramine-T (5 mg/mL) were added, and the mixture was incubated for 1 hour at room temperature. Sodium disulfite (20 μL; 10 mg/mL) was added, and after incubation at room temperature for 5 minutes, the mixture was applied to a Bio-Gel P-20 column equilibrated with PBS and eluted with PBS. The specific activities of ^{125}I-labeled Tf ranged from 60 to 65 cpm/fmol. Confluent cell monolayers were incubated for 24 hours in serum-free DME/F12 medium, rinsed with Tf binding buffer (25 m*M* HEPES, 0.9 m*M* $MgCl_2$, 0.15 *M* NaCl, 1 mg/mL bovine serum albumin [BSA], pH 7.5), and incubated in Tf binding buffer for 20 minutes at 4 °C. ^{125}I-labeled Tf was added, and the cells were incubated for various times at 4 °C. After the incubation with ^{125}I-labeled Tf, the cells were washed six times with ice-cold Tf washing buffer, and cell-associated radioactivity was determined after solubilization in 25 m*M* HEPES, 1% (vol/vol) Triton X-100, and 1 mg/mL BSA. Nonspecific binding of ^{125}I-labeled Tf was estimated by labeling in the presence of a 200–1000-times excess concentration of unlabeled Tf (*32*).

Scatchard Analysis of ^{125}I-Labeled Tf Binding

Confluent cell monolayers were incubated for 2 hours at 4 °C with ^{125}I-labeled Tf in the presence or absence of 200-times excess unlabeled Tf in 5 mL of labeling buffer. At the end of incubation (2 hours), the cells were rinsed six times with the Tf washing buffer without BSA, and unbound Tf was determined by gamma-scintillation counting. The cells were then solubilized with 1% (vol/vol) Triton X-100, 10% (vol/vol) glycol, 25 m*M* HEPES, 1 mg/mL BSA, pH 7.5, and cell-bound Tf was determined as described (*32*).

Effect of Anti-Tf on Lung Tissue-Conditioned Medium

Fractionated goat antihuman transferrin (Sigma Chemical Co., St. Louis, Mo.) and normal goat IgG (Sigma Chemical Co.) were biotinylated using sulfo-NHS biotin. Crude rat lung tissue-conditioned medium (LCM) was made 0.35 mg/mL in biotinylated antitransferrin or 5 mg/mL in biotinylated goat IgG and incubated overnight at 4 °C. To each milliliter of treated medium was added 300 μL of a 50% slurry of streptavidin-agarose (BRL). After a 30-minute incubation, the agarose was removed by centrifugation at 2000*g* for 5 minutes, and the supernatants were dialyzed against 25 m*M* HEPES, pH 7.5, overnight. The dialysates were made equimolar in medium components by the addition of 1/9 volume of 10 × medium, filtered sterilized, and 5, 10, or 15 μL was added to MTLn3 cell cultures (2000 cells per well in a 96-well plate) in 100 μL α-MEM medium containing 0.3% FBS. Cell numbers were quantitated 4 days later using a crystal violet staining procedure where A_{590} correlates closely with cell number (*27*). The addition of FBS to these assays was performed so as to duplicate the culture conditions made when the mitogenic effects of LCM were initially observed and the responsive component were uncharac-

terized. At this level of FBS, cell viability was maintained, but proliferation was slow to nil.

Statistical Analysis

Data were analyzed by one-way or two-way analysis of variance and considered significantly different from the indicated groups when $P < .05$.

RESULTS AND DISCUSSION

Metastatic Tumor Cell Growth Responses to Organ Tissues, Tissue-Conditioned Media, or Extracts from Tissues

Previously, Hart (*2*) found that extracts of kidney, lung, and spleen could stimulate the tissue culture growth of lung-colonizing murine B16 melanoma and liver-colonizing murine M5076 monocytic tumor cells. Hart (*2*) did not find a correlation between the abilities of these cell lines to metastasize to their target organs in vivo and their response to the organ extracts in vitro. Mouse lung extracts were slightly stimulatory to both lung-colonizing B16-F10 melanoma and liver-colonizing M5076 cells, but liver extracts inhibited the growth of both cell lines. In contrast to Hart (*2*), Yamori et al (*33*) reported that a mouse lung extract increased the serum-free growth in culture of three murine colon carcinoma lines of high lung-metastasizing ability. Moreover, the extract stimulated the growth of only one of three poorly lung-metastasizing colon carcinoma lines. This investigation concluded that a positive growth response to a lung growth factor was one of the important properties of lung-metastatic colorectal tumor cells.

Several investigators have used the conditioned medium obtained from organs or organ tissue fragments to investigate whether organ (paracrine) growth factors might be involved in the organ preference of metastasis. Horak et al (*17*) examined the effect of normal syngeneic tissue fragments in the same culture as tumor cells or tissue-conditioned medium added to tumor cells on the growth of 52 separate spontaneous murine mammary carcinoma cell lines. The presence of lung tissue fragments or the addition of lung-conditioned medium stimulated the growth of most of the cell lines. This was in contrast to ovary-, liver-, thyroid-, and kidney-conditioned media that inhibited the growth of most of the mammary carcinoma cells. Because the preferred site for metastasis of most of these lines is the lung, the positive growth response to lung-conditioned medium correlated with metastasis to lung. At the same time, we were measuring the ability of conditioned media from lung, ovary, liver, kidney, and brain to stimulate or inhibit the in vitro growth of murine B16 melanoma sublines previously selected to preferentially colonize lung, ovary, or brain (*16*). Lung-conditioned medium preferentially stimulated the growth of the lung-metastasizing cell lines but also had a slight proliferative effect on the other lines. Ovary-conditioned medium stimulated the growth of lung- and ovary-colonizing cells but not brain-colonizing cell sublines. Liver-conditioned medium had a pronounced inhibitory effect on all of the B16 cell sublines. Kidney tissue-conditioned medium at low concentrations stimulated the growth of lung- and ovary-metastasizing B16 sublines but at higher concentrations inhibited the proliferation of all of the B16 sublines. Brain-conditioned medium showed a slight stimulation of the growth of lung-metastasizing sublines and had a slight inhibitory effect on brain and ovary targeting sublines. This pattern of growth stimulation and inhibition indicated that response to organ-associated growth factors might be important in the organ preference of metastasis (*16*).

Other studies performed in our laboratory have investigated the growth-stimulating activity of kidney-, brain-, liver-, and lung-conditioned media on murine RAW117 large-cell lymphoma cells that are poorly metastatic (RAW117-P) or highly metastatic cell sublines sequentially selected to colonize liver (RAW117-H10) or lung and liver (RAW117-L17) (*18*). Whereas kidney or brain tissue-conditioned medium inhibited or had no effect on the growth of any of the RAW117 cell lines, liver tissue-conditioned medium inhibited the growth of RAW117-P cells but stimulated the growth of the highly liver-metastatic H10 and L17 sublines. Lung tissue-conditioned medium stimulated the growth of all RAW117 lines at low concentrations, but at higher concentrations this tissue-conditioned medium preferentially stimulated the growth of H10 and L17 sublines (*18*).

Similar results were obtained with epithelial tumor systems. We found that clones of the rat 13762NF mammary adenocarcinoma that metastasize spontaneously to the lung (such as MTLn3) proliferated in culture in low serum-containing medium in the presence of rat or porcine lung tissue-conditioned medium (*19*). A subline (MTC) derived from the same parental tumor but of poor lung-metastasizing ability responded to a lesser degree to the medium from various organ tissues, or it did not respond at all (*19*).

The presence of specific organ tissues in culture can also enhance the growth of metastatic cells. Naito et al (*34*) monitored the survival and growth of poorly and highly lung-metastatic murine K-1735 melanoma cell sublines and liver-metastasizing murine M-5076 reticulum sarcoma cells in cultures containing organ tissue pieces or tissue-conditioned medium. They found a correlation between the lung-colonizing potentials of tumor cells and their ability to survive in the presence of lung tissue fragments in vitro. They also found that the tumorigenic properties of lung-metastatic sublines were enhanced when the cells were co-injected with lung tissue fragments. The lung- and liver-metastasizing sublines grew in culture on the lung tissue fragments, but only the liver-metastasizing cells grew in association with the liver tissue fragments. Using lung tissue-conditioned medium to stimulate the growth of the tumor cell sublines Naito et al (*34*) found that the lung tissue-conditioned medium contained high levels of growth-stimulating activity for lung-colonizing cells but only low levels of activity for liver-colonizing cells (*34*).

The growth of tumor cells in the presence of specific organ tissue pieces has been used as a method to select malignant cell sublines in vitro for increasing growth rate, metastatic potential, and organ preference of colonization. Price et al (*35*) found that sequential selection of poorly lung-colonizing B16-F1 murine melanoma cells for growth in low serum-containing medium on lung or kidney tissue fragments resulted in selection of a highly lung-colonizing variant subline. Although the melanoma cells selected to grow on lung or kidney tissue fragments developed increased lung-colonizing potential, those selected to grow on kidney tissue fragments were incapable of colonizing the kidney. Subcloning of the cells selected for growth in the presence of lung tissue fragments resulted in sublines of various growth potentials in the presence of lung tissue fragments. The lung colonization potential of these clones, however, did not always correlate with their ability to grow with the lung tissue fragments, indicating that other factors (probably cell adhesion, cell motility, and cell invasion, among others [*3–5*]) are also necessary for increased organ preference of colonization.

Metastatic Tumor Cell Growth Responses to Insoluble Tissue-Derived Growth Factors

In addition to soluble growth factors released from organ tissues in culture, the presence of cell contact-dependent mitogens in organ tissues could be important in stimulating growth of organ-metastasizing tumor cells. These mitogens probably include extracellular matrix and plasma membrane-bound growth factors, matrix components themselves, and other relatively insoluble growth factors or integral plasma membrane components (*5, 36–42*). For example, extracellular matrix components can modify the growth properties of some tumor cells (*38–40*), and growth factors can be found complexed to integral membrane proteoglycans (*41*). Doerr et al (*22*) found that extracellular matrix prepared from various organs differentially stimulated metastatic cell growth at low cell densities. They found that highly metastatic rat mammary carcinoma and human hepatoma cells were growth stimulated better than poorly metastatic cells by organ matrix prepared from the target organ metastatic colonization. Fractionation of extracellular matrix from the organ matrix preparations revealed that a glycosaminoglycan fraction was the most active growth regulator (*22*). Although highly metastatic cells responded differently to extracellular matrix heparan sulfate, Redini et al (*43*) did not find a clear-cut relationship between the growth response of various rhabdomyosarcoma tumor and myoblast cell lines to heparan sulfate and their metastatic properties. In general, they found that the most metastatic lines were the least inhibited by heparan sulfate (*43*). The differential stimulation of metastatic cell growth by insoluble growth factors displayed by organ parenchymal cells has been studied by Sargent et al (*36*). They found that a B16 melanoma subline sequentially selected to colonize liver proliferated more rapidly when co-cultured with normal murine hepatocytes than the original parental B16 line. The same parental line was also selected to colonize the lung, but the lung-colonizing variant did not possess the increased growth response when co-cultured with hepatocytes. The growth-stimulating activity was not attributed to soluble factors released from the hepatocytes. Glutaraldehyde fixation of the hepatocytes abolished the growth-stimulatory activity, and cell-cell contact appeared to be required for B16 cell growth stimulation.

We have found that a lung particulate fraction obtained from a rat lung homogenate that was freed of all soluble and peripheral membrane proteins differentially stimulated the growth of highly lung-metastatic tumor cells. The insoluble growth-promoting material was prepared by several exposures of lung tissue to a chaotropic buffer followed by ultracentrifugation. The resulting pellet was treated with a CHAPS (3-[3-cholamidpropyl]-dimethylammonio-1-propanesulfonate) detergent solution to solubilize the mitogenic activity for lung-metastasizing rat mammary tumor cells. This aqueous-insoluble but CHAPS-soluble fraction contained mitogenic activity for highly spontaneous metastatic rat MTLn3 mammary adenocarcinoma cells but not for their nonmetastatic counterpart line MTPa. The mitogenic activity was not found in a chloroform-methanol extract of the detergent-solubilized material, nor was it affected by trypsin treatment, bound by immobilized heparin, or affected by high temperatures (95 °C for 1 hour). The mitogenic activity was affected, however, by chemical reduction. Removal of CHAPS detergent by dialysis resulted in partial loss of solubility and activity. The responsible component eluted from QAE-Sepharose at high salt concentrations >1.0 *M* NaCl at pH 8.5) and migrated with an M_r >200 000 on a Bio-Gel P-200 gel filtration column. Analysis of partially purified material by SDS-polyacrylamide gel electrophoresis revealed major silver-stained bands of M_r ~95 000, ~115 000, and ~200 000 (*37*).

Purification of Soluble Organ (Paracrine) Growth Factors for Highly Metastatic Tumor Cells

Some of the mitogens secreted by organ tissues that differentially stimulate the growth of highly metastatic tumor cells have been purified or partly purified. Szanlawska et al (*44*) found that lung tissue-conditioned medium contained a growth-stimulating activity for both normal and tumor cells. Separation of the activity by gel filtration demonstrated that it possessed an M_r ~50 000–70 000. Yamori et al (*33*) used gel filtration of lung tissue-conditioned medium to partially purify a lung-derived tumor cell mitogen. The mitogenic activity had an M_r of 90 000–120 000, and the activity was partially destroyed by exposure to 56 °C or 80 °C or totally inactivated by trypsin treatment.

Using rat or porcine lung tissue-conditioned medium, we extensively purified a lung mitogen for highly lung-metastatic rat MTLn3 mammary adenocarcinoma cells (*26,27*). Purification of the activity from the lung tissue-conditioned medium by hydroxylapatite affinity chromatography, anion exchange chromatography, chromatofocusing, gel filtration, and preparative native gel electrophoresis resulted in a pure preparation of a growth-

stimulating glycoprotein for lung-metastasizing tumor cells. We named this mitogen lung-derived growth factor-1 (LDGF-1). This protein was of M_r ~66 000 (in unreduced gels) and M_r ~72 000 (in reduced gels), and it had a pI of 6.9–7.0. The mitogen also had a fairly low specific activity (ED_{50} for MTLn3 cells of 0.3–0.5 μg/mL) (*26*). The mitogenic activity of LDGF-1 was abolished by exposure to high temperature (95 °C for 1 hour) or treatment with reducing agents (*27*). Using different animal tumor metastatic systems where cell sublines of poorly and highly lung-metastatic potential were available, we assayed the mitogenic activity of LDGF-1. This mitogen differentially stimulated the growth of highly lung-metastatic epithelial, lymphoid, and mesenchymal tumor cells. For example, LDGF-1 differently stimulated the highly metastatic sublines of murine B16 melanoma (Fig. 1, A), rat 13762NF mammary adenocarcinoma (Fig. 1, B), and murine RAW117 large-cell lymphoma (data not shown).

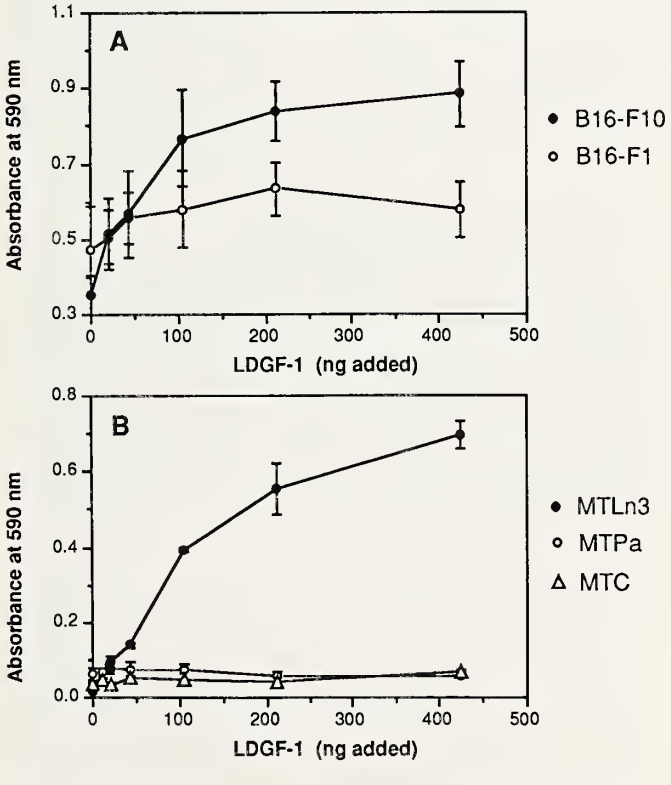

Fig. 1. Dose-response of murine B16 melanoma and rat 13762NF mammary adenocarcinoma cell lines and clones to purified LDGF-1. The results were obtained when increasing amounts of purified porcine LDGF-1 were added to cell cultures (plated at a density of 2000 cells/well in 96-well plates) with medium containing 1% (vol/vol) fetal bovine serum. One day later the medium was replaced with fresh medium containing 0.3% fetal bovine serum, and the amount of LDGF-1 indicated was added to respective wells. Four days later, the relative cell numbers in each well were quantitated using a crystal violet staining procedure that correlates A_{590} with cell number (*27*). A: Results obtained with murine B16 melanoma variants of lung-metastasizing capability: B16-F10 > B16-F1. B: Results obtained with rat 13762NF mammary adenocarcinoma clones of lung metastasizing capability: MTLn3 > MTPa, MTC. Data are the mean ± SD of four replicates.

We also found that LDGF-1 was active in stimulating the growth of human MCF-7 and other breast cancer cell lines (data not shown). The mitogenic activity of LDGF-1 was not inhibited by antibodies against several known growth factors, such as insulin, granulocyte-macrophage colony stimulating factor, platelet-derived growth factor, or epidermal growth factor, suggesting that LDGF-1 was not antigenically related to these known growth factors.

Identification of LDGF-1 as a Tf-Like Protein

Although our initial studies did not reveal any similarity of LDGF-1 to known growth factors, further characterization of LDGF-1 indicated that it was a member of the transferrin (Tf) family of growth factors, or it was a Tf-like molecule (*45*). The similarities of LDGF-1 to Tf were demonstrated by the nearly identical migration of rat and porcine LDGF-1 compared to human and rat Tf in SDS-polyacrylamide gels, cross-reactivity of antiporcine LDGF-1 with human and rat Tf, and cross-reactivity of antihuman Tf with rat LDGF-1. In addition, the M_r of polypeptides obtained after trypsin cleavage were identical for porcine LDGF-1 and porcine Tf.

Because we had identified LDGF-1 as a Tf-like molecule, we examined the ^{125}I-Tf binding properties and growth response to Tf of tumor cell sublines of different metastatic properties. In the murine B16 melanoma system, the brain-colonizing B16-B15b subline exhibited the greatest growth response to Tf, followed in order by the ovary-colonizing B16-O13, the highly lung-colonizing B16-F10, and finally the poorly lung-colonizing B16-F1 subline (*32*). Because the B15b and O13 lines colonize the lung as well as the brain or ovary (*46*), we expected these B16 sublines to respond to Tf. Cell binding of ^{125}I-Tf to the B16 cell sublines paralleled their growth responses to Tf. Although the differences in quantitative ^{125}I-Tf binding to the different B16 sublines were not as great as the differences in growth responses among the B16 sublines, the rank order of ^{125}I-Tf binding and growth responses were similar (B15b > O13 > F10 > F1) (*32*).

Using the rat mammary adenocarcinoma metastatic system, we also found a relationship between the binding of ^{125}I-Tf and growth responses to Tf and spontaneous metastatic potential. The high brain- and lung-metastasizing sublines (PaB10 and PaB5) showed the highest binding of ^{125}I-Tf; cell binding was five to six times higher than any of the other lines. The highly lung-metastasizing MTLn3 line bound less ^{125}I-Tf than the brain-metastasizing lines, followed by the intermediate lung metastasizing MTF7 and the poorly lung-metastasizing MTPa line (Fig. 2). We calculated the numbers of Tf receptors on these cell lines by analysis of Scatchard plots obtained when increasing concentrations of ^{125}I-Tf were allowed to bind to cell monolayers. The results indicated that receptor numbers increased as spontaneous metastatic capability increased in the following order: high brain-metastasizing ability (~150 000 receptors/cell) greater than high lung-metastasizing ability (~70 000 receptors/cell) greater than poor metastatic capability (~35 000 receptors/cell; Fig. 3). It is interesting that two different affinities for Tf were

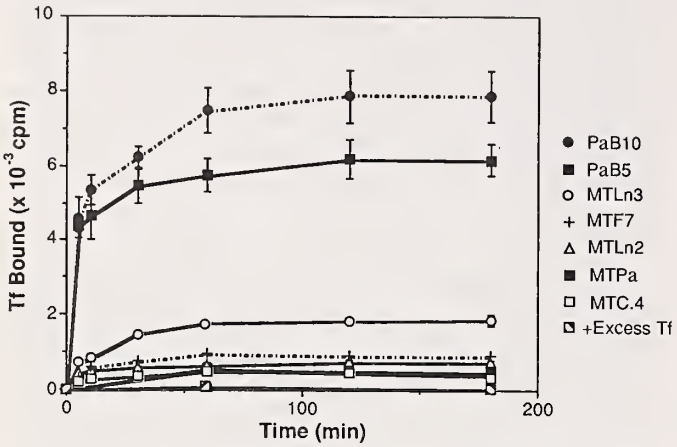

Fig. 2. Time course of binding of ^{125}I-Tf to monolayers of various sublines of the rat 1376NF mammary adenocarcinoma. Cultures were established in 12-well plates, placed in serum-free conditions for 24 hours, equilibrated to 4 °C in binding buffer (25 m*M* HEPES, 0.9 m*M* $MgCl_2$, 0.15 *M* NaCl, 1 mg/mL bovine serum albumin [BSA], pH 7.5), and allowed to bind ^{125}I-Tf as described in the Materials and Methods section. Cells were then washed with BSA-free binding buffer, solubilized, and counted.

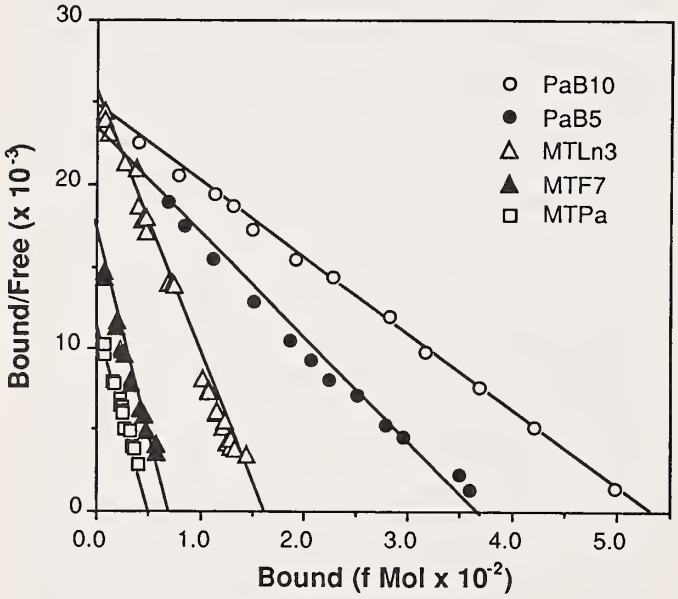

Fig. 3. Scatchard analysis of ^{125}I-Tf binding to various rat 13762NF mammary adenocarcinoma sublines. Holo rat transferrin was radioiodinated using the chloramine T procedure. Cells were grown to confluency in 12-well plates and placed in fetal bovine serum-free conditions for 24 hours prior to assay. Monolayers were washed and equilibrated at 4 °C in 25 m*M* HEPES, pH 7.5, containing 150 m*M* NaCl, 1 m*M* $CaCl_2$, 1 m*M* $MgCl_2$, and 1% (wt/vol) bovine serum albumin. Increasing amounts of ^{125}I-Tf were added to duplicate wells and incubated at 4 °C for 2 hours. Monolayers were washed and solubilized with 1% NP-40 detergent, and total bound radioactivity was determined. Data are the mean of two wells. In all cases, bound radioactivity could be reduced to background by the addition of excess cold Tf.

seen: the brain colonizing lines possessed K_d values of 17–22 fmol, whereas the K_d values for the other lines were 4.7–6.0 fmol. We have examined several human breast cancer cell lines established from brain or lung metastases for the presence of Tf receptors and response to Tf. The results indicate that human tumor cells may also respond to Tf similar to the way animal metastatic tumor systems respond. Although the true biologic potential of the human breast cancer cell lines in the syngeneic host is not known, the human breast cancer cell lines with potential to metastasize in nude mice were the ones that expressed high numbers of Tf receptors. Thus, our data using animal metastatic tumor systems may be highly relevant to human cancers.

To demonstrate that a Tf-like activity in lung tissue-conditioned medium was responsible, in part, for the stimulation of mammary adenocarcinoma cell growth, we used a specific anti-Tf biotinylated antibody to remove Tf and Tf-like molecules from lung tissue-conditioned medium. When lung tissue-conditioned medium was treated with control biotinylated goat antibodies and streptavidin-agarose, growth activity was not removed; however when the lung tissue-conditioned medium was treated with biotinylated goat anti-Tf and streptavidin-agarose, there was a significant reduction ($P < .001$) in the growth potential of the lung tissue-conditioned medium (Fig. 4).

Overexpressed Tf Receptors and Enhanced Response to Tf in Organ Preference of Metastasis

Although there are other important growth factor responses, the ability of malignant cells to proliferate in certain tissue compartments may be facilitated by their expression of Tf receptors and response to paracrine secretion of Tf. We found that one of the most potent growth factors obtained from organ-conditioned medium is a Tf or Tf-like molecule (*26,27*). We also found that the most metastatic cell sublines of murine melanoma and rat mammary adenocarcinoma were the most responsive to Tf in growth factor-limiting medium and, in general, expressed the highest numbers of Tf receptors. Tumor cells that express high numbers of Tf receptors should be able to respond to low, limiting concentrations of Tf that exist in some tissue compartments, such as the brain. In organs such as brain, Tf is probably used as a paracrine growth factor during development (*47*). With the possible exception of the choroid plexus, uninjured adult brain does not synthesize Tf, and Tf is normally present in low quantities, probably because of its poor penetration through the blood-brain barrier. Thus, for malignant cells to metastasize to sequestered compartments, such as the brain, it may be advantageous for them to express high numbers of Tf receptors and respond to low concentrations of Tf.

Relationship of Metastatic Cell to Normal Cell Growth Responses

Malignant cells can respond to many well-known and characterized growth factors. The ability to proliferate in the presence of certain of these factors correlates with

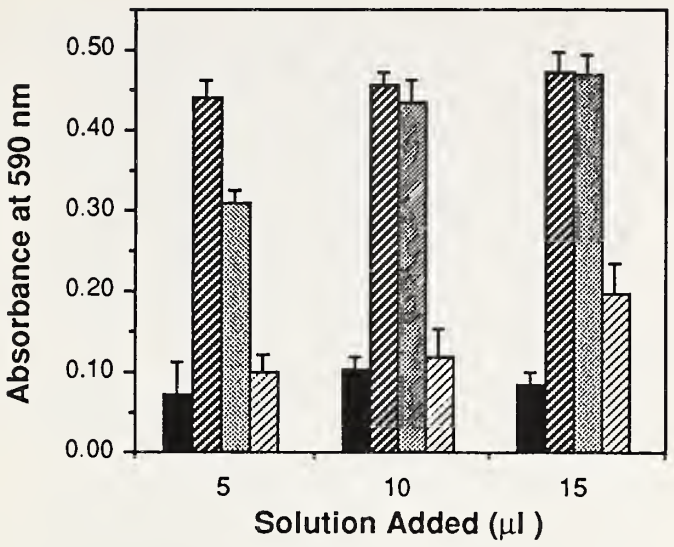

Fig. 4. Effect of anti-Tf on the growth of rat MTLn3 cells stimulated by lung tissue-conditioned medium. Increasing amounts of biotinylated normal goat IgG or biotinylated goat antihuman transferrin were added to lung tissue-conditioned medium (LCM). After a 30-minute incubation, the streptavidin-agarose was removed by centrifugation at 2000*g* for 5 minutes, and the supernatants were dialyzed overnight, made equimolar in medium components, and added to MTLn3 cell cultures (2000 cells/well in 96-well plates) containing α-MEM and 0.3% FBS. Cell numbers were quantitated 4 days later using a crystal violet staining procedure. Results are compared to those obtained using control medium only or untreated LCM. *Bars* = mean ± SD of three replicates.

metastatic properties in some systems (*30,48*). Most of the studies examining the effects of various growth factors on metastatic cells did not assess the possibility that any of these factors might exist at differing concentrations in the target sites for metastatic colonization. Tumor cell growth responses to crude extracts or tissue-conditioned medium from organs at high risk for metastasis indicate that these growth responses correlate with the ability to metastasize to particular sites. Characterization of these (paracrine) growth factors has not revealed new or novel organ-associated growth factors that could be responsible for the differential growth of metastatic cells. We have found that the major growth-promoting molecule from lung tissue-conditioned medium for lung-metastasizing tumor cells is Tf or a Tf-like glycoprotein (*27,44*). Whether increased response to Tf is a tumor cell attribute that is required for lung metastasis remains to be shown, but it is extremely unlikely that only one such factor can determine metastatic properties (*5,6*).

Metastatic cell response to target organ-associated growth factors is probably only one of a number of tumor cell properties required for the successful formation of metastases. Although highly liver-metastatic murine RAW117-H10 cells respond well to lung tissue-conditioned medium, they fail to colonize the lungs of their syngeneic host. In this case, the highly liver-metastatic RAW117 cells show very poor rates of adhesion to lung-derived microvessel endothelial cell and rapid rates of clearance through the lungs when injected intravenously into mice (*9*). Thus it should be obvious that additional properties, such as adhesion to microvessel endothelial cells in the target organ, secretion of degradative enzymes, response to chemotactic and haptotactic motility factors, and other properties are collectively important in determining whether a malignant cell can successfully colonize a particular site (*4–6*).

In considering the growth factor-dependence of tumor cells capable of organ preference of metastasis, phenomena such as tissue damage incurred by the attachment and invasion of tumor cells into the target organ of metastasis would be expected to result in the release of growth factors similar to those released during tissue wounding. High responsiveness of malignant cells to these factors might result in the successful growth and formation of a new secondary tumor (*49*).

Finally, the identification of (paracrine) growth factors that can differentially stimulate the growth of metastatic cells at particular organ sites could lead to new therapeutic strategies for the treatment of metastases. The Tf receptor has already been the focus of therapeutic strategies. Scott et al (*50*) have used toxin-conjugated anti-Tf receptor to kill human cancer cells expressing the Tf receptor in nude mice, and Elliot et al (*51*) have found that platinum-Tf complexes can inhibit the metastasis of rat mammary adenocarcinoma cells in vivo. As additional (paracrine) growth factors are discovered, new therapeutic approaches could increase our success in treating established metastases.

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Expression of Prohormone Processing Enzymes in Neuroendocrine and Non-Neuroendocrine Cells[1]

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ABSTRACT—The biosynthesis of many peptides thought to have autocrine or paracrine effects on cell growth requires a series of enzymatic steps. The level of expression of two of these posttranslational processing enzymes was compared in several endocrine and non-neuroendocrine cell lines. Peptidylglycine α-amidating monooxygenase (PAM; EC 1.14.17.3) is a bifunctional copper- and ascorbate-dependent enzyme essential in the formation of α-amidated peptides. Carboxypeptidase H (CPH; EC 3.4.17.10) removes basic residues from the carboxy-terminus of the products of endoproteolytic cleavage of prohormones and is generally essential in the formation of substrates for PAM. PAM messenger RNA (mRNA) and activity were detectable in both endocrine (AtT-20, GH_3) and non-neuroendocrine (L, 3T3, COS, BRL, C127) cells. Except for BRL cells, CPH mRNA and enzymatic activity were detectable in all the cell lines. BRL cells contained no detectable CPH mRNA and had a different carboxypeptidase B-like activity. Thus, expression of secretory granule-associated processing enzymes is not limited to cells of a classic neuroendocrine phenotype. [J Natl Cancer Inst Monogr 13:163–168, 1992]

The conversion of preprohormones into their product peptides involves a series of co- and posttranslational modifications that occur sequentially in the endoplasmic reticulum, Golgi complex, and secretory granules (*1,2*). The enzymes catalyzing early steps in this pathway, such as signal peptide cleavage, disulfide bond formation, and protein *N*-glycosylation, are common to the endoplasmic reticulum and Golgi complex of most cell types. Enzymes catalyzing later steps in the pathway, for example, specific endoproteolytic cleavages, α-*N*-acetylation, and α-amidation, have generally been considered specific to neuroendocrine cells. Many of the peptides thought to exert effects on cell growth through autocrine or paracrine mechanisms (gastrin-releasing peptide, vasopressin, tachykinins, gastrin) terminate with a COOH-terminal α-amide (*3,4*). Production of α-amidated peptides from their precursor molecules generally involves the sequential action of an endoprotease specific for paired basic amino acids, removal of newly exposed COOH-terminal Lys or Arg residues by carboxypeptidase H (CPH), and conversion of the resultant peptidyl-Gly intermediate into the des-glycine peptidyl-α-amide by peptidylglycine α-amidating monooxygenase (PAM). Peptide precursors terminating with a COOH-terminal Gly can be acted upon directly by PAM (*1,2,5–7*) (Fig. 1). Ascorbic acid is generally the most effective co-substrate for the first reaction catalyzed by the bifunctional PAM enzyme, although some cell types can substitute other reducing agents for ascorbic acid (*1,2*).

In the course of screening cell lines as potential hosts for the exogenous expression of different alternatively spliced forms of PAM messenger RNA (mRNA), it became clear that expression of PAM was not limited to neuroendocrine cells. Since PAM cannot function in the absence of CPH, we decided to determine whether expression of both enzymes was more widespread than previously anticipated. AtT-20 mouse corticotropic tumor cells store ACTH, β-endorphin, and other peptides produced from the pro-ACTH/endorphin precursor in secretory granules and are routinely used as model neuroendocrine cells in studies of posttranslational processing and regulated secretion (*8*). Production of growth hormone requires signal peptidase cleavage but does not require additional endoproteases, CPH, or PAM; nevertheless, GH_3 cells expressing pro-ACTH/endorphin or prosomatostatin process these foreign prohormones into products that require the presence of all of these enzymes (*8*). Both AtT-20 cells and GH_3 cells were previously shown to contain PAM and CPH mRNA and activity (*1,8*). The expression of PAM and CPH in three fibroblast lines (L cells, 3T3 cells, and COS cells), a mammary epithelial cell line (C127 cells), and a hepatoma line (BRL cells) was compared to their expression in these two classical neuroendocrine cells. PAM and CPH expression were quantitated by measurement of mRNA levels and enzyme activity.

MATERIALS AND METHODS

Cell Lines

All cell lines were maintained in a 1:1 mixture of DMEM:F-12 containing penicillin-G (0.12 mg/mL), streptomycin sulfate (0.2 mg/mL), and glutamine (0.6 mg/mL). Mouse AtT-20/D-16v cells were maintained in

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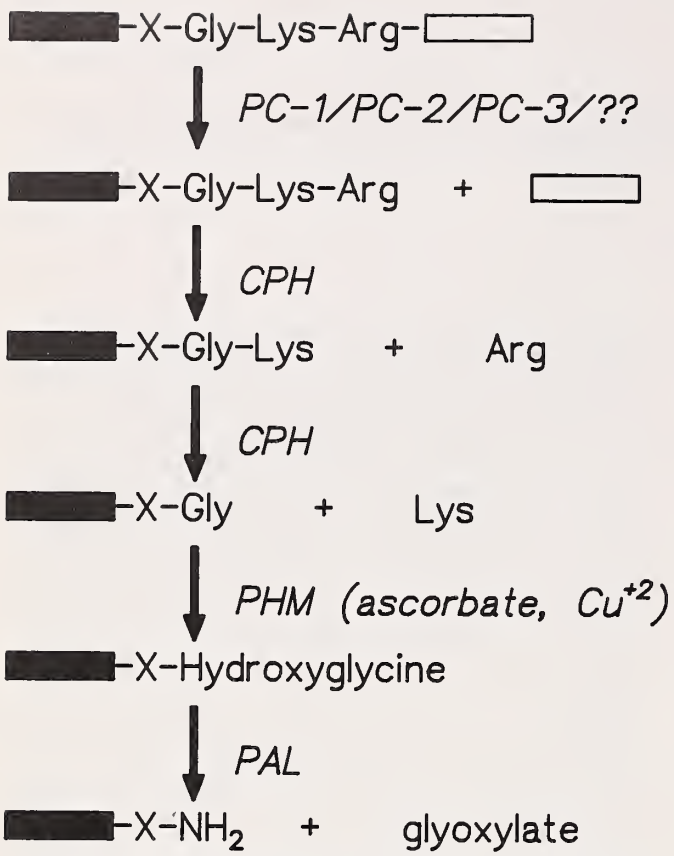

Fig. 1. Steps in the biosynthesis of α-amidated peptides. Endoproteolytic cleavages commonly occur at pairs of basic amino acids or at single Arg residues; Lys-Arg is the pair of basic residues found most commonly. Endoproteolytic cleavage may also occur between the pair of basic amino acids. PCs, prohormone converting enzymes, are subtilisin-like endoproteases thought to cleave prohormones (*5,6*). PHM, peptidylglycine α-hydroxylating monooxygenase, and PAL, peptidyl-α-hydroxyglycine α-amidating lyase, are both encoded by the bifunctional PAM precursor and can function independently (e.g., in the pituitary) or as part of a bifunctional protein (e.g., heart and MTC cells) (*1,2*). *Filled* and *open rectangles* denote peptide segments preceding and following the processing site.

medium containing 10% horse serum, 10% fetal bovine serum (Gibco) and 10% Nu-Serum (Collaborative Research). Rat GH_3 cells were obtained from Dr. Carter Bancroft (Mt. Sinai School of Medicine) and were maintained in medium containing 15% horse serum and 2.5% fetal bovine serum. Buffalo rat liver BRL 3A cells (ATCC CRL 1442), mouse connective tissue L-M (TK-) cells (ATCC CCL 1.3), mouse NIH-3T3 cells (Swiss albino embryo fibroblasts; ATCC CCL 92), and mouse mammary tumor C1271 cells (ATCC CRL 1616) were maintained in medium containing 10% fetal bovine serum. Monkey kidney fibroblast COS cells were obtained from Dr. Keith Peden (Howard Hughes Medical Institute, The John Hopkins University School of Medicine) and were maintained in medium containing 10% fetal bovine serum and 10% Nu-Serum. Cells were fed with fresh medium approximately 24 hours before harvest and were routinely grown until confluent, when samples were to be harvested for RNA or enzyme analysis.

Analysis of RNA

RNA was extracted from cell pellets with guanidine thiocyanate (*9*). RNA concentrations were estimated by measuring absorbance at 260 and 280 nm, and 10 μg of RNA was routinely fractionated on denaturing agarose gels; samples were passively transferred to Nytran, baked, prehybridized, and hybridized (10^6 cpm/mL) as described (*9*). Probes used include the 1.3 kb *Pstl/Bam*HI fragment of rat PAM-1 (bp 351-1681) (*10*), the 0.7 kb *Eco*RI/*Eco*RI fragment of bovine PAM (bp 782-1503) (*10*), and the 2 kb *Eco*RI/*Eco*RI fragment of the pSP-65 rat CPH vector (provided by Lloyd Fricker, Albert Einstein College of Medicine) (*9,11*). For quantitation, blots were stripped and rehybridized with ribosomal RNA complementary DNA (cDNA) fragments (*9*). Autoradiograms were densitized with a LOATS RAS-1000 Image Analysis system (Amersham), and corrections were made for film non-linearity. Signals for PAM and CPH mRNA were normalized to the amount of ribosomal RNA.

Analysis of Enzyme Activity

Cells were washed twice in pre-warmed HEPES-buffered medium and scraped from the dish. For assay of PAM activity, cell pellets were suspended in 20 m*M* Na TES, 10 m*M* mannitol (pH 7.0) containing protease inhibitors and frozen before dispersion with a Polytron (*12*). The crude nuclear pellet was resuspended in the initial volume of 20 m*M* Na TES, 10 m*M* mannitol (pH 7.0) by homogenization and stored frozen. The supernatant from the low-speed centrifugation was separated into a soluble fraction and a washed crude particulate fraction by centrifugation as described (*12*). The crude particulate fraction and crude nuclear pellets were solublized by incubation with 1% Triton X-100 before assay. PAM assays were routinely carried out at 37 °C for 4 to 8 hours using 0.5–3 μg protein per 40 μL assay with [^{125}I]-D-Tyr-Val-Gly, 0.5 μ*M* D-Tyr-Val-Gly, 10 μ*M* $CuSO_4$, 100 μg per mL catalase, 0.50 m*M* ascorbate, in 125–150 m*M* Na TES (pH 8.5) (*12*). For each cell line, the specific activity of PAM in the crude nuclear, crude particulate, and soluble fractions was determined. Total specific activity was calculated by summing the PAM activity in all three fractions and dividing by the sum of the protein in all three fractions.

For assay of CPH, cell pellets from duplicate 60-mm dishes of each cell type were individually resuspended in 100 μL of 100 m*M* Na acetate (pH 5.6) and 0.3 mg/mL phenylmethylsulfonyl fluoride and frozen (*13,14*). Samples were homogenized as above, frozen again, and cell debris was then removed by centrifugation at 8500*g* for 5 minutes. This low-speed supernatant was used for all CPH assays. Duplicate aliquots of each sample (1 to 4 μg protein per 100 μL assay) were assayed with [^{3}H]-benzoyl-phenylalanine-alanine-arginine (New England Nuclear, 30 Ci/mmol; approximately 5×10^4 dpm/assay tube), 5 μ*M* benzoyl-phenylalanine-alanine-arginine (Peninsula Labo-

ratories, Inc., Belmont, Calif.), 100 m*M* Na acetate (pH 5.6) in the presence of 1 m*M* $CoCl_2$ or in the presence of 1 m*M* $CoCl_2$ plus 1 μM guanidino-ethylmercaptosuccinic acid (GEMSA) as described (*13,14*). After incubation at 37 °C for 1 hour, the reaction was stopped by addition of 50 μL 1 N HCl, and the product was extracted into $CHCl_3$, dried, and quantitated by liquid scintillation spectrometry. CPH activity was taken to be the amount of $CoCl_2$-stimulated activity that was inhibited by GEMSA (*13–15*). Samples were also assayed with 50 m*M* Tris HCl (pH 7.5) substituted for the standard acetate buffer. Duplicate assays of the same sample agreed within an average of plus or minus 3%. Protein concentrations were determined using the bicinchoninic acid reagent (Pierce Chemical Co., Rockford, Ill.) with bovine serum albumin as standard.

RESULTS

Expression of PAM

Northern blot analysis of total RNA prepared from each of the different cell lines revealed the presence of an approximately 4 kb mRNA hybridizing with probes for rat and bovine PAM (Fig. 2A). As seen by comparing the signal obtained when the same blot was probed sequentially with rPAM cDNA and bPAM cDNA, quantitative comparisons of relative amounts of PAM mRNA in the different cell lines must be confined to cell lines derived from the same species. Based on the analysis of at least two independent preparations of RNA from each of the cell lines, the neuroendocrine AtT-20 cells, which produce and secrete an α-amidated product peptide, contained amounts of PAM mRNA similar to the amounts in two mouse fibroblast lines (Fig. 2); normalized to ribosomal RNA, 3T3 cells contained 60% ± 10% and L cells 190% ± 40% as much PAM mRNA as contained in AtT-20 cells. The mammary epithelial cells contained 180% ± 30% as much PAM mRNA as in AtT-20 cells. The BRL hepatoma cells contained 37% ± 6% as much PAM mRNA as in the neuroendocrine GH_3 cells. Evaluated with the bPAM probe, the COS monkey kidney fibroblast line contained as much PAM mRNA as the neuroendocrine cells. Although the various cell lines contained readily detectable levels of PAM mRNA, the amount of PAM mRNA in the atrium of the rat heart exceeded that in the GH_3 cells by approximately 100-fold (Fig. 2B).

When extracts of the individual cell lines were assayed for amidation activity using D-Tyr-Val-Gly at pH 8.5, all contained detectable levels (Fig. 3). This assay detects PHM, the first enzyme contained within the bifunctional PAM protein, since the alkaline pH obviates the need for the second enzyme, PAL (Fig. 1). Since subcellular fractionation left a large percentage of the total PAM activity and total protein in the crude nuclear pellet for many of

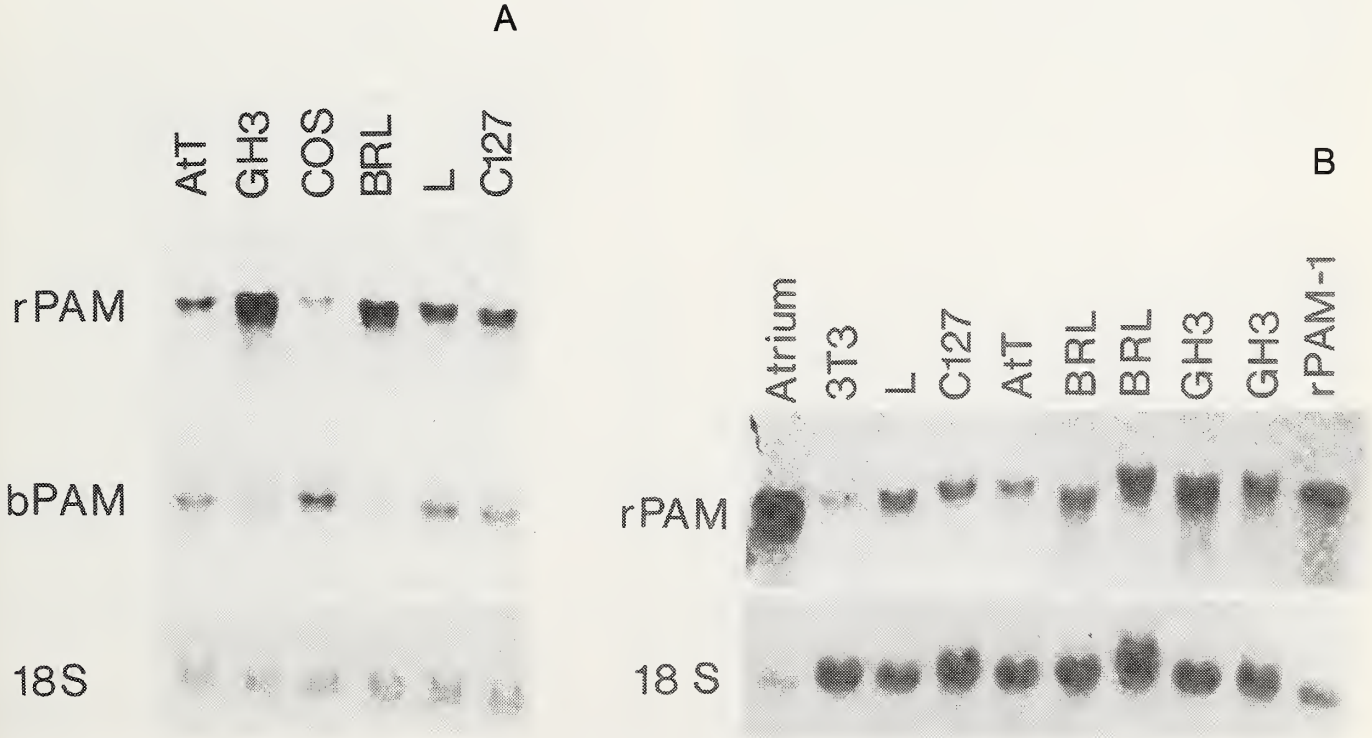

Fig. 2. Identification of PAM mRNA. *Left panel.* Aliquots of total RNA (10 μg) from the cell lines indicated were subjected to Northern analysis. Blots were exposed first to the rat PAM probe, then to the bovine PAM probe, and finally to the ribosomal RNA probe (18S). *Right panel.* RNA (10 μg) from the cell lines indicated and from adult rat heart (0.5 μg) was fractionated as above, and the blot was hybridized with the rPAM probe followed by the ribosomal RNA probe. The rPAM standard represents 100 pg of an in vitro transcript prepared from a plasmid containing rPAM-1 cDNA; liver RNA was added as carrier (*16*).

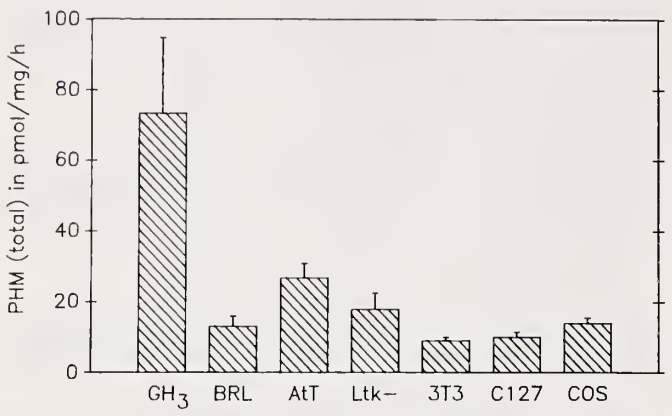

Fig. 3. PAM activity. Crude nuclear, crude particulate, and soluble fractions were prepared at least three times from each cell line. Since the assays were carried out with D-Tyr-Val-Gly at pH 8.5, they most closely reflect PAM activity. Typically, cells from 10 60-mm dishes were harvested each time. Assays were carried out in duplicate with at least two different amounts of protein. The mean total specific activities ± SEM are plotted.

the cell lines, total PAM specific activity was calculated by summing the amount of PAM activity in the crude nuclear, crude particulate, and soluble fractions (Fig. 3). The two neuroendocrine cell lines consistently contained higher levels of PAM activity than the other cell lines, with levels in extracts of GH_3 cells significantly higher than in extracts of AtT-20 cells. None of the cell lines contained levels of PAM activity comparable to those in rat anterior pituitary (430 pmol/mg per hour) or rat atrium (1500 pmol/mg per hour) (*16*). PAM-specific activity in the hepatoma, mammary carcinoma, and fibroblast lines was 30% to 50% of that in the neuroendocrine AtT-20 line, but activity was readily detectable. The distribution of PAM activity among the soluble and crude particulate fractions was cell-type specific. Approximately 80% of the PAM activity in the three fibroblast lines examined was found in the crude particulate fraction. In the GH_3, BRL, AtT-20, and C127 cells, 40% to 50% of the PAM activity was found in the crude particulate fraction. Subsequent studies have indicated that several of these cell lines secrete substantial amounts of PHM and PAL activity into the medium.

Detection of Carboxypeptidase H

The presence of CPH in these various cell lines was determined by Northern blot analysis (Fig. 4). A cDNA probe encompassing most of the protein coding region of rat CPH visualized an approximately 2.1 kb mRNA in all the cell lines examined with the exception of the BRL cells. When normalized to the amount of ribosomal RNA present, the C127 cells contained almost as much CPH mRNA (90% ± 10%) as AtT-20 cells. Both of the mouse fibroblast lines examined contained significant levels of CPH mRNA; averaged from three separate preparations of RNA, 3T3 cells contained 27% ± 7% and L cells 46% ± 7% of the level of CPH mRNA found in AtT-20 cells.

Four separate preparations of RNA from BRL cells were examined and in none was a signal detected with the rat CPH cDNA probe (Fig. 4). The integrity of these RNA preparations was demonstrated by the detection of undegraded PAM mRNA in the same preparations of BRL RNA (Fig. 2). The GH_3 cells contained at least 30-fold more CPH mRNA per ribosomal RNA than did the BRL cells and approximately eight-fold more CPH mRNA per ribosomal RNA than the heart atrium.

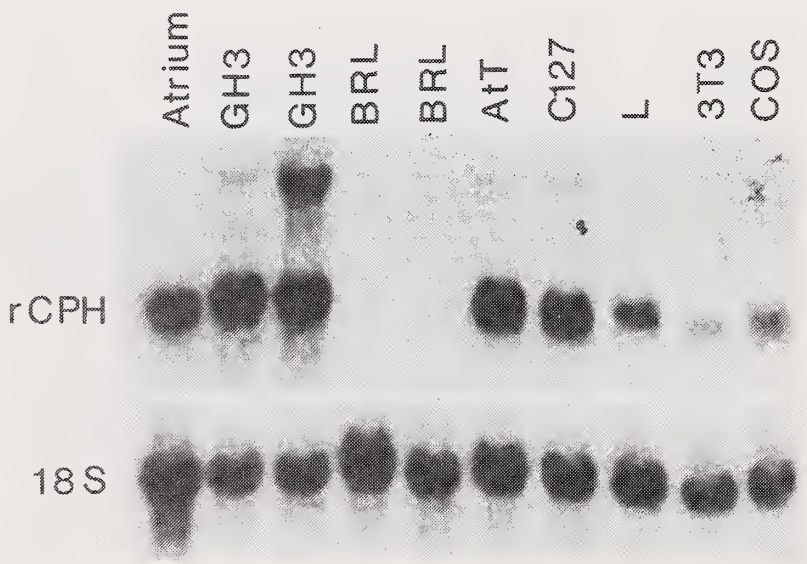

Fig. 4. Identification of CPH mRNA. Total RNA (10 μg) from the cell lines indicated and from adult rat atrium (10 μg) was fractionated as described in Fig. 2. The blots were hybridized with the rat CPH probe followed by the ribosomal RNA probe (18S).

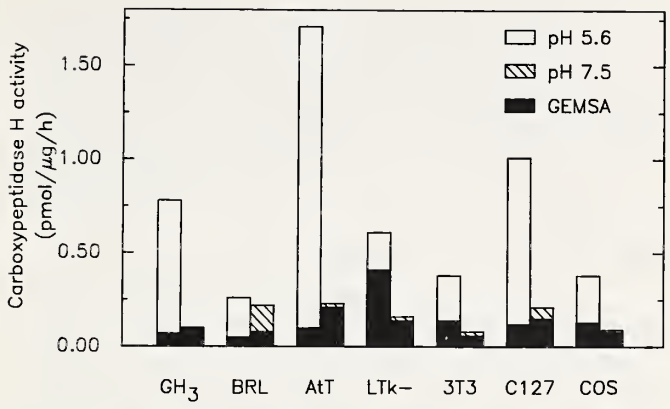

Fig. 5. CPH activity. CPH activity was measured in cell extracts in the presence of exogenous $CoCl_2$; data were obtained at pH 5.6 and at pH 7.5 in the absence and presence of GEMSA. CPH activity was defined as GEMSA-blockable, cobalt-stimulated activity measured at pH 5.6.

The lack of CPH mRNA in BRL cells was unexpected; absence of a secretory granule-associated carboxypeptidase B-like enzyme would limit the ability of these cells to generate substrates for PAM from most prohormone molecules. Therefore, enzyme assays were carried out to quantitate levels of CPH-like activity using [^{3}H]benzoyl-phenylalanine-alanine-arginine as substrate (Fig. 5). Taking the amount of $CoCl_2$ stimulated and GEMSA blockable carboxypeptidase activity at pH 5.6 as a measure of CPH activity (*13–15*), high levels of CPH were found in AtT-20 cells, C127 cells, and GH_3 cells. These same cell lines displayed high levels of CPH mRNA. The fibroblast lines studied contained significant amounts of $CoCl_2$-stimulated, GEMSA-blockable activity, although the specific activity was about sixfold lower than in AtT-20 cells.

Surprisingly, extracts of BRL cells assayed under the same conditions contained 30% as much $CoCl_2$-stimulated, GEMSA-blockable carboxypeptidase activity as GH_3 cells. Because no CPH mRNA could be detected in BRL cells, the properties of this activity were further investigated by carrying out the carboxypeptidase assay at higher pH; CPH exhibits almost no activity at pH 7.5 (*7, 17*). BRL extracts exhibited 63% as much $CoCl_2$-stimulated, GEMSA-blockable activity at pH 7.5 as at pH 5.6, suggesting that the cells contain an enzyme distinct from CPH. In all other cell types examined, the amount of $CoCl_2$-stimulated, GEMSA-blockable activity observed at pH 7.5 was approximately 10% of the level observed at pH 5.6.

DISCUSSION

Significant levels of expression of two prohormone posttranslational processing enzymes, PAM and CPH, have been measured in a variety of cell lines not previously recognized as competent to process prohormones. Although the level of PAM activity in AtT-20 cells is 10-fold lower than in the anterior pituitary (*16*), over 90% of the joining peptide produced by AtT-20 cells maintained in plasma levels of ascorbate is α-amidated (*18*). Therefore the level of PAM activity in the other cell lines should be high enough to produce significant amounts of α-amidated product if the appropriate preprohormone, endo- and exo-proteases, and reducing equivalents are available. As our ability to detect α-amidated peptides increases, substrates for the action of these enzymes in non-neuroendocrine cells will likely be identified. For example, many cell types produce insulin-like growth factors (IGFs) (*19*); the IGF-IB precursor contains two potential endoproteolytic cleavage sites whose use would generate a substrate for CPH and PAM (*4,20,21*). Similarly, mammary epithelial cells produce parathyroid hormone-related peptide; this prohormone contains a potential α-amidation site in a highly basic region near its COOH-terminus (*22*).

Although GH is not subject to endoproteolytic cleavage and is not α-amidated, the presence of significant levels of CPH and PAM in GH_3 cells could be inferred from the fact that expression of transfected mouse pro-ACTH/endorphin and proneuropeptide Y in GH_3 cells resulted in the efficient production of α-amidated joining peptide and neuropeptide Y (*23,24*). In addition, wild type GH_3 cells secrete a VIP-like peptide (VIP is α-amidated) with autocrine actions on PRL secretion (*25*).

The lack of CPH mRNA in BRL cells is consistent with the fact that normal hepatocytes produce carboxypeptidase N and contain almost no CPH or CPM mRNA (*11,26–28*). The CPB-like enzymatic activity in BRL cells exhibits properties similar to those of CPN (*25*). In contrast, Hep G2 human hepatocarcinoma cells contain more CPH than CPN (*27*). BRL-3A cells synthesize pro-IGF-II and cleave this precursor into smaller product peptides (*29*), but a role for PAM in these cells remains to be identified.

With improved techniques for studying the post-translational processing enzymes identified in tissues producing and storing large amounts of product peptide for secretion, it is clear that expression of these enzymes is not limited to classic neuroendocrine cells. For example, the enzyme cleaving proalbumin to albumin in hepatocytes is remarkably similar to one of the two enzymes responsible for cleaving proinsulin in insulin secretory granules (*30*). In several studies, fibroblast cell lines (3T3 and COS) transfected with expression vectors encoding preprohormones (PTH and somatostatin) were found to be capable of carrying out limited endoproteolytic processing (*31,32*). This more widespread distribution of the enzymes required for the generation of bioactive peptides normally stored in secretory granules suggests an even more extensive use of peptides as autocrine and paracrine messengers. By understanding the enzymes and cofactors involved in their biosynthesis, it may be possible to better control production of the bioactive forms of these peptides.

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Control of Tumor Cell Biology Through Regulation of Peptide Hormone Processing[1,2]

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ABSTRACT—Control of the biology of individual cells, organs, and organisms is achieved through an interplay of a host of specific interactions, many involving peptide hormones as modulators or effectors. In tumor cells these processes may result in uncontrolled growth as a consequence of autocrine and/or paracrine growth effects. Most peptide hormones are bioactive only after processing of the precursor prohormone by posttranslational processing enzymes. The enzymes involved with the production of bioactive peptides, in particular those associated with peptide α-amidation, provide potential targets for disruption of posttranslational processing mechanisms as a means of regulation of the progression of tumor cells. This information provides a rational basis for consideration of inhibition of autocrine neuropeptide synthesis as a strategy for chemointervention. [J Natl Cancer Inst Monogr 13:169-175, 1992]

Our group has been working with peptide hormones since the identification of gastrin-releasing peptide (GRP) as a tumor marker and an autocrine growth factor of small-cell lung cancer cells. We have described a GRP-neutralizing monoclonal antibody that blocks autocrine growth stimulation by GRP and is being tested in a phase II clinical trial (*1,2*). GRP is a peptide hormone that is α-amidated at the carboxy terminus, as are approximately half of all known neuroendocrine peptide hormones, including several other autocrine and paracrine growth factors (*3*). α-Amidation is essential for the bioactivity of most amidated peptides, including GRP, and we are therefore exploring the biology of peptide amidation to identify new potential sites for disruption of autocrine stimulation. We will review the biochemistry of the enzymes responsible for the posttranslational production of α-amidated peptide hormones from inactive prohormones and show how substrate selectivities and microenvironment requirements could provide targets for control of posttranslational processing in tumor cells.

The complex cell-cell and tissue-tissue interactions of organisms are often regulated by peptide hormones. In normal cells, specificity of interactions mediated by peptide hormones often derives from cell-specific and tissue-specific production of these hormones (*4,5*), although instances of differential expression of receptors in target cells have also been reported (*6*). These interactions are often subsumed by neoplastic cells where the normal peptide hormone ligand/receptor interactions can result in autocrine and paracrine growth-stimulatory signals, for example: the small-cell lung cancer autocrine growth factor GRP functions as a gastrointestinal neuropeptide in normal adult biology. Blockade of peptide hormone/receptor interactions, either by anti-hormone or anti-receptor antibodies, has been used in a number of preclinical and clinical trials for control of autocrine tumor growth biology (*2,7*). Similar strategies using peptide hormone antagonists have also been proposed. However, it is possible that heterogeneity of tumor cell autocrine mechanisms within any given tumor, as well as the capacity of some tumor cells to respond to multiple autocrine factors, could limit the utility of this approach as a general chemointervention tool. We have, therefore, explored additional strategies for neutralization of autocrine growth factor effects in cancer promotion by studying the common mechanisms by which many peptide hormones are synthesized. These mechanisms may provide potential sites for simultaneous interruption of multiple autocrine pathways.

The production of a peptide hormone such as GRP is a multistep process. A general scheme of the pretranslational and posttranslational processes involved in production and secretion of a peptide hormone is diagramed in Fig. 1. The events depicted are only indicative of processes that may occur for a peptide hormone. The order in which the processes occur can also vary between peptides. For instance, there are reports of endoproteolytic processing occurring in the Golgi apparatus before vesicule sorting, yet certain endoproteolytic steps must occur after sorting because they are dependent upon prior modifications of the prohormone for site specificity (*8–10*).

Many of the processes shown in Fig. 1 have an established role in regulating cell biology in nature. Pretrans-

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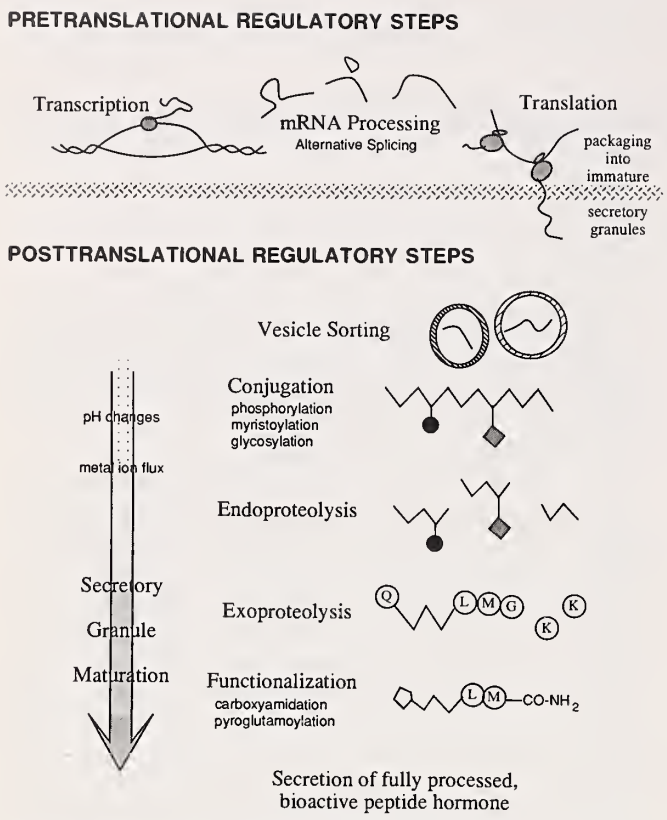

Fig. 1. Known and potential regulatory steps in the production and secretion of a peptide hormone.

lational events are loci for control of diversity of peptide hormone production, as in the case of the mRNAs for calcitonin and calcitonin gene-related peptide (CGRP), which are generated from various combinations of the six exons of their common gene (*11*). Alternate splicing of these exons to produce either calcitonin or CGRP mRNA is controlled in a tissue-specific manner and leads to a variety of effects due to the differing activities of the two peptide hormones. Although the mechanisms are not yet clear, posttranslational processes are also subject to tissue-specific regulation. Adrenocorticotrophic hormone, which is released from pro-opiomelanocortin (POMC) in both the anterior and intermediate pituitary lobes by proteolysis at . . .LysArg. . . sequences at both termini, has an internal . . .LysArg. . . sequence that is cleaved only in the intermediate pituitary (*12*). Furthermore, extensive and precise posttranslational processing is often required to create an active peptide hormone from its prohormone. Posttranslational modifications such as α-amidation in many cases have been shown to be essential for proper biological function (*13,14*). Several avenues of recent research suggest that the processes of posttranslational modification are also subject to cell- and tissue-specific regulation.

Posttranslational processing of prohormones can be considered as two types of reactions: those that affect the length of amino acid polymers but do not modify the chemistry of the amino acid sequence, the endo- and exoproteolytic processing reactions, and those reactions that do modify the chemistry of amino acids by creating posttranslational modifications (Table 1). Posttranslational modifications can be further divided into two groups of reactions: conjugation and functionalization. The amino acid modifications shown in Table 1 are examples that have been well documented, but new posttranslational modifications are reported frequently.

The first-described, and currently best-understood, posttranslational amino acid modification is C-terminal amidation. α-Amidation was first reported in 1953 as a structural feature of the mammalian hormones oxytocin (*15,16*) and arginine-vasopressin (*17*). C-terminal amidation, ie, the presence of an α-carboxyamido group instead of a free carboxylic acid on the amino acid at the C-terminus of a peptide, is now known to occur on approximately half of the recognized mammalian neuronal and endocrine peptide hormones, including gastrin, GRP, cholecystokinin, thyrotropin releasing hormone, calcitonin, growth hormone releasing factor, vasoactive intestinal peptide, and gonadotropin releasing hormone. The presence of a C-terminal carboxyamido amino acid on novel peptides is not only accepted as de facto proof of biological activity but has also been used as a criterion for identification of novel peptide hormones (*13,18,19,* Siegfried JM, Kasprzyk PG, Treston AM, submitted for publication).

Fig. 2A shows the steps leading to formation of the C-terminal methionineamide of GRP. The sequence . . .GlyLysLys. . ., which is found in the GRP prohormone, is representative of a motif for peptide amidation. The only amino acid that is invariant in the α-amidation process is the glycine. A review of amidated peptides and their precursors can be found in Cuttitta et al (*3*). As shown in Fig. 2A, posttranslational synthesis of α-carboxyamidated peptides generally requires three enzymatic activities, endopeptidases, exo- (carboxy) peptidases, and the glycine-directed peptidyl α-amidating activity. The amide group of the peptide hormone is formed by oxidative cleavage of the glycine-extended prohormone (*20*). As detailed in Fig. 2B, peptidyl α-amidation is the

Table 1. Posttranslational processing steps

Modify length of amino acid polymer	Modify peptide and amino acid chemistry
Endopeptidase	*Conjugation*
Signal peptide cleavage	Phosphorylation
Cleavage at basic residues	N-,C-, and S-acetylation
	Glycosylation
Exopeptidase	Sulfation
Aminopeptidase	
Carboxypeptidase	*Functionalization*
	C-terminal α-amidation
	Gln cyclization
	Lys, Pro hydroxylation
	Asp, Glu γ- and β-carboxylation

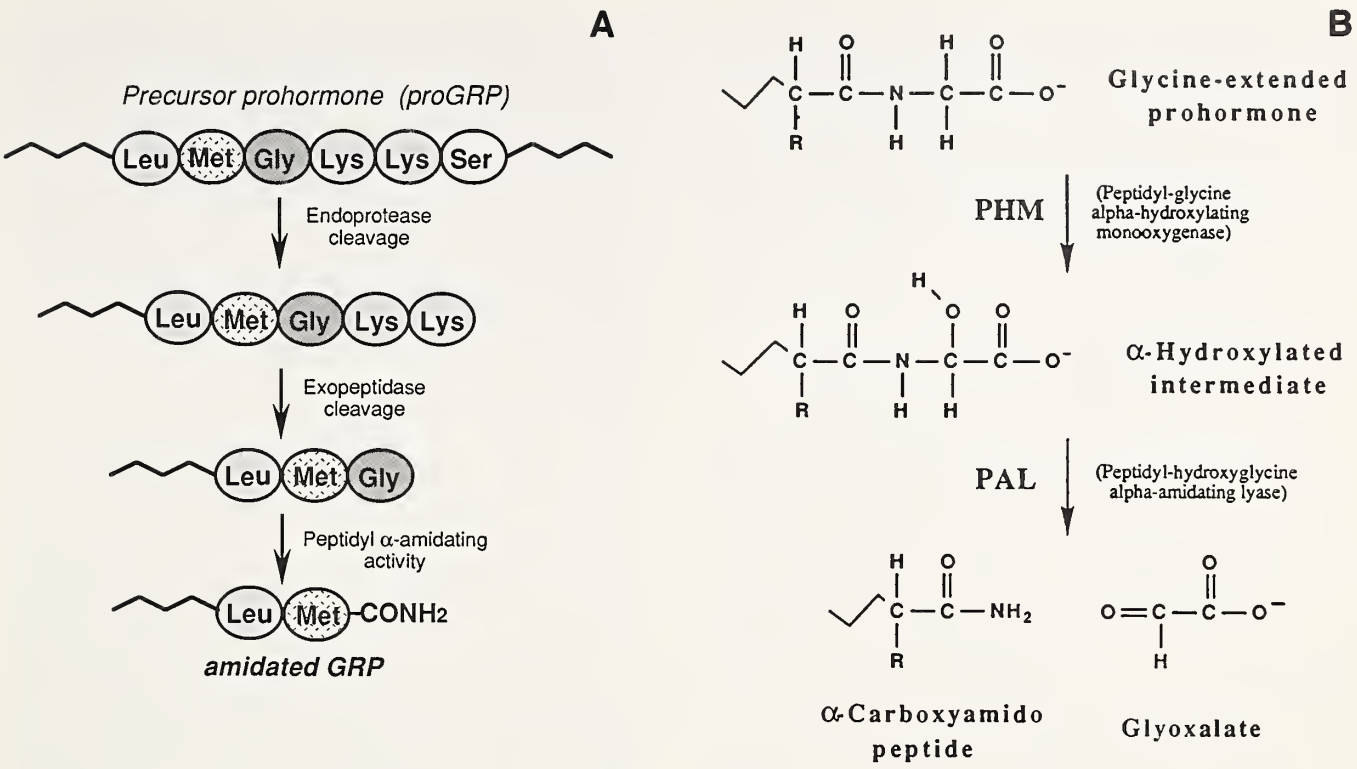

Fig. 2. A: Sequence of endoproteolytic, exoproteolytic, and functionalization posttranslational processing reactions that convert the pro-carboxy-terminal region of proGRP into the bioactive α-amidated hormone. B: Detailed mechanism of the two enzyme activities responsible for the posttranslational processing reaction, peptidyl C-terminal α-amidation.

overall reaction catalyzed by two sequential enzymes: peptidyl-glycine α-hydroxylating monooxygenase (PHM) and peptidyl-α-hydroxyglycine α-amidating lyase (PAL) (*21,22*). Because α-amidation is usually essential for the bioactivity of amidated peptides, these four enzyme activities must all be present and functional for efficient synthesis of bioactive amidated peptide hormones from their prohormones. A number of reports suggest that normal cells may control posttranslational processing through these enzymes.

The most important general mechanisms available for a cell to control prohormone processing appear to be 1) synthesis of multiple enzymes with similar functions but varying specificities and 2) regulation of the microenvironment of the secretory granule through pH control and copackaging of prohormones, enzymes, cofactors, and inhibitors. These general mechanisms are both relevant to control of the enzymes involved with peptide α-amidation. Other mechanisms that in some instances may be important include spatial separation (vesicle sorting) of parallel processing pathways (*23*) and differences in the temporal order of successive modifications [reviewed in (*10*)]. Correct processing of prohormone precursors in a particular cell is the outcome of a complex relationship among enzyme specificity, enzyme inhibitors and cofactors, and secretory granule microenvironment. Potentially, any factor that affects the accuracy of posttranslational processing enzymes could have important biological effects. Therefore these control mechanisms could provide targets for novel therapeutic interventions in tumor cells.

The steps in posttranslational processing that are the most conspicuous targets for antiprocessing intervention are those for which the biochemistry of the enzymes is the best understood. It has only recently become possible to study the endoproteases involved in posttranslational processing as the molecular sequences of several candidate enzymes have been proffered (*24*). A number of enzymes capable of endoproteolytic prohormone processing had previously been described by Marx (*25*). It is not yet clear whether the biochemical characteristics of what were called the "tryptic-like enzymes" or "prohormone converting enzymes" will apply to the newly described subtilisin-like endopeptidases. The basic amino acid exopeptidases (carboxypeptidases) and the glycine-directed α-amidating complex, in contrast, are now apparently reasonably understood. Some carboxypeptidases have been localized to prohormone-containing secretory granules and have been shown to have substrate selectivities and pH activity profiles consistent with secretory granule precursor processing, reviewed by Fricker (*26*). These enzymes include the Lys- and Arg-specific carboxypeptidases B and E (also called carboxypeptidase H) and a bovine histidine-specific carboxypeptidase. As yet no

mammalian carboxypeptidase has been definitively linked to the production of any particular peptide hormone, although in vitro they cleave basic amino acids from model substrates and basic amino acid-extended peptides. To date only a single enzyme activity responsible for C-terminal amide formation has been characterized. Few studies on the newly described second enzyme in this pathway (PAL, Fig. 2B) have been carried out. However both the first enzyme, PHM, and a bifunctional PAM (PHM+PAL) enzyme from a variety of tissues and organisms have been well studied (*21*). Cancer cell lines that express a variety of markers of the neuroendocrine phenotype express both PHM and PAL activities (*27,28*).

There are marked differences between the cofactor requirements for these processing enzymes. Metal ions are essential cofactors for the carboxypeptidase and peptidyl α-amidation enzymes. Davidson and Hutton (*29*) have described a carboxypeptidase that is specific for Co^{2+}; PHM and PAM are dependent on and highly selective for Cu^{2+} (*27,30*) and PAL is a less specific metalloenzyme (*28,31*). Another demonstrated cofactor of PAM and PHM is ascorbate, and a correlation between ascorbate levels and production of amidated peptides in several endocrine tissues has been suggested (*32*). Dietary deprivation of ascorbate has little effect on ascorbate levels in these tissues, suggesting that a homeostatic mechanism maintains co-factor levels in cells responsible for production of amidated peptides.

These biochemical requirements of posttranslational processing enzymes suggest that tumor cells dependent on autocrine stimulation could be growth-inhibited by removal of these cofactors. The effects of divalent cation deficiency on prohormone processing have been noted in vitro. Dog pancreatic islet cells grown in media lacking Cu^{2+} correctly cleave the pancreatic polypeptide prohormone and remove the basic residues at the resultant C-terminus but release a glycine-extended form of the peptide instead of the biologically active C-terminal amidated hormone (*33*). Treatment of cells both in vitro and in vivo with specific copper chelators (disulfiram and diethyldithiocarbamate) has been shown to reduce the amidation of murine Joining Peptide (*34*). This effect was linked in that report to inhibition of the PHM step in glycine-directed peptidyl α-amidation. We have preliminary results that suggest that the small-cell lung cancer cell line NCI-H345, known to be dependent on GRP for autocrine-stimulated growth, is growth-inhibited in the presence of disulfiram (Fig. 3A). This inhibition is not seen with the same concentrations of disulfiram in the non-endocrine lung tumor cell line A549 (Fig. 3C). The growth inhibition of NCI-H345 has not yet been directly linked to a decrease in the levels of bioactive amidated GRP in the medium.

Another approach that could be applied to inhibit posttranslational processing enzymes is use of specific substrate analogs. Peptide analogs of the substrates of the processing enzymes would inhibit by simple competitive mechanisms, but covalent irreversible inhibitors may be of more use in a clinical setting. Potent inhibitors of most posttranslational processing enzyme involved with amidation are known, for example, phenylmethylsulfonylfluoride (endoproteases), guanidinoethylmercaptosuccinic acid (carboxypeptidases), and 4-phenyl-3-butenoate (PHM). The lipophilic non-peptide molecule 4-phenyl-3-butenoate irreversibly inhibits PHM in vitro and has been reported to enter cells and covalently inhibit PHM in secretory granules (*35*). Treatment of GRP-dependent NCI-H345 tumor cells with 4-phenyl-3-butenoate resulted in growth inhibition (Fig. 3B). As with disulfiram, this effect was not seen with A549 cells (Fig. 3D). It will be essential to carry out detailed studies on pure enzymes to develop new inhibitors of all posttranslational processing enzymes and determine which inhibitors are most selective for the enzymes involved in posttranslational processing of autocrine growth factors in tumor cells. Current reports on the endoprotease enzymes suggest that there may be one enzyme, the product of the fur gene, that is responsible for cleavage of protein precursors in the constitutive pathways of cells and a family of enzymes that cleaves prohormones in the regulated secretory pathway, pc2 and pc3 (*24,36,37*). Such a division leaves open the possibility of specifically inhibiting prohormone processing while sparing cleavage of constitutive precursors. Further fine-tuning of such an approach might be possible if selective inhibitors could be designed for different members of the families of processing enzymes. For example, the production of a number of bioactive peptides from the somatostatin prohormone has been shown to be related to the presence of at least two endoproteases (*38*) and at least two members of the subtilisin-like endoprotease family are known, pc2 and pc3 (*36,39*). Similarly, multiple forms of both PHM and PAM enzymes have been found in tumor cell extracts (Treston AM, manuscript in preparation). The significance of these multiple enzyme species with respect to potential inhibitor selectivity awaits purification of large amounts of each isoenzyme.

Some other facets of the secretory granule microenvironment have also been reported to play a role in control of prohormone processing enzymes. These include endogenous inhibitors of processing reactions and chemical microenvironmental factors such as pH. Reported endogenous inhibitors of prohormone processing reactions include proteins specifically copackaged in the secretory granule such as chromogranin A (*40*) and products of the processing reactions themselves that act through feedback inhibition (*41*). Because many of the enzymes described above have acidic pH optima, they are most active at the pH of secretory granules. However, the pH of these organelles is reported to rise as the granules mature (*42*), so it is likely that the processing enzymes are in an inactive state in the Golgi apparatus and immature granules and that pH changes coupled to specific cofactor ion fluxes during granule maturation determine their activities (*43–45*). Possible therapeutic strategies based on a more complete understanding of posttranslational processing mechanisms could therefore include inhibition through stimulation of synthesis of endogenous inhibitors such as chromogranin A, through nonhormonal peptide

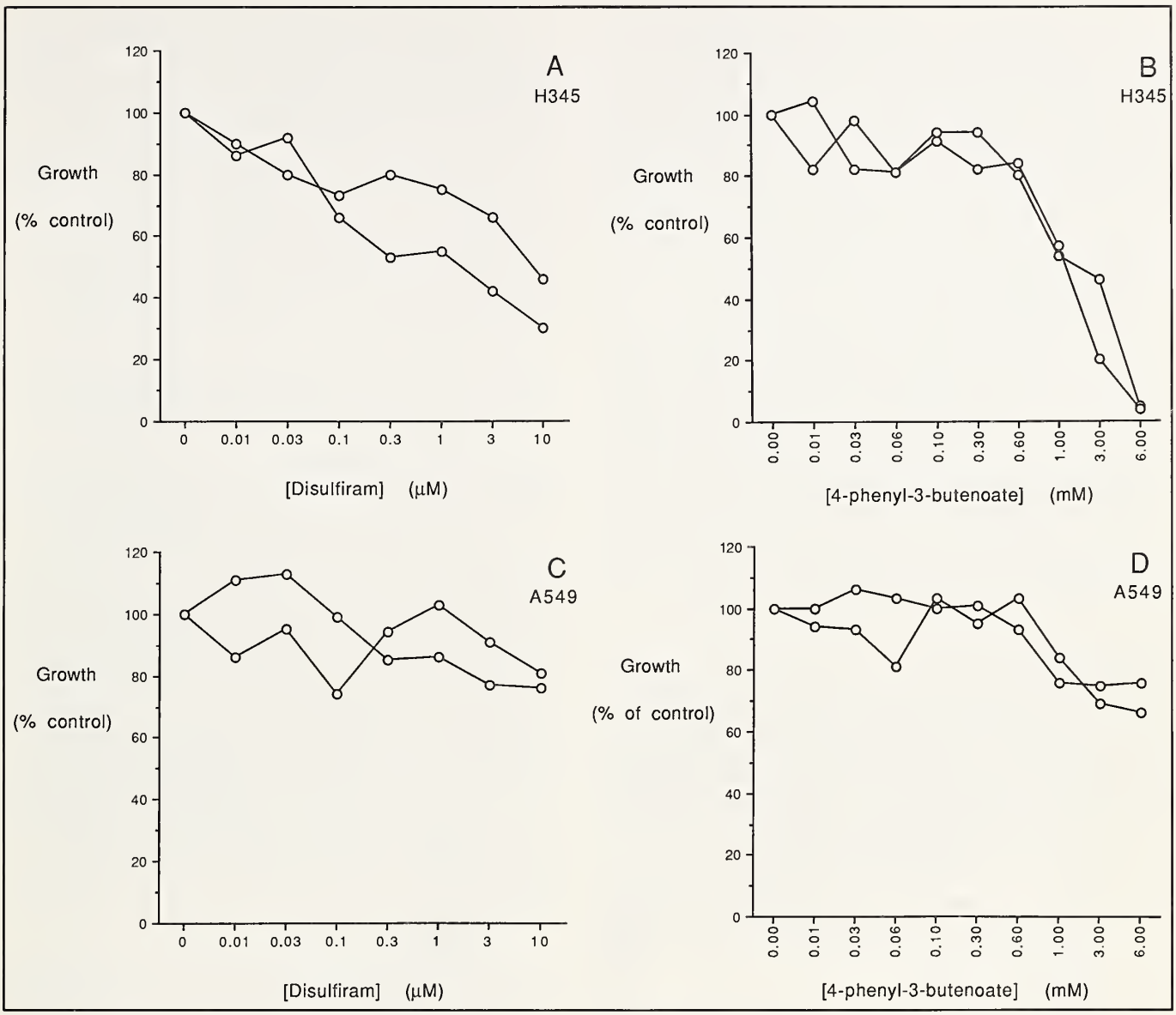

Fig. 3. A: Growth of NCI-H345, determined by MTT assay, in the presence of disulfiram. B: In the presence of 4-phenyl-3-butenoate. C: Growth of A549, determined by MTT assay, in the presence of disulfiram. D: In the presence of 4-phenyl-3-butenoate. Results of two separate experiments for each cell line and treatment are shown. Standard deviations have been omitted for clarity.

substrates introduced to the cell using transfection or systemic treatment, and through inhibition of pH-dependent processes in secretory granules using agents such as chloroquine, as well as inhibition of metallo-enzymes with specific metal chelators and inhibition with non-peptide substrate analogs as already described. It may also be possible to induce incorrect processing by activating transcription of inappropriate processing enzymes, or by transfection of cells with DNA coding for processing enzymes with inappropriate substrate specificities.

Although targeting a particular processing enzyme or neuroendocrine process in a tumor cell could have undesirable effects on non-neoplastic endocrine cells in the patient, tumor cells may be more susceptible to interventions of this type due to their rapid growth and possible reliance on autocrine growth factors that may not be involved in growth regulation of normal cells. This situation applies to small cell tumor cells, on which GRP acts as an autocrine growth factor, although GRP in the normal adult is present principally as a gut and brain neurotransmitter. As described above, it may prove possible to use studies on the substrate and cofactor specificities of posttranslational processing enzymes to select inhibitors that affect particular pathways of neoplastic cells and, thus, spare similar pathways in normal cells. Strategies for regional delivery of anti-processing agents could also be used to mitigate systemic toxicity. Coupling of anti-prohormone processing agents to tumor-specific anti-

bodies could produce a marked increase in specificity of delivery compared to systemic treatment with the same agent. Early detection of tumor may also provide the opportunity to utilize therapeutic avenues that would be problematic if applied systemically in patients with advanced disease. Detection of endocrine lung cancer when it is confined to the inner membrane of the bronchus would allow delivery of anti-prohormone processing agents to the bronchial space without the risks of systemic application of the agents. Similar considerations apply to many of the anti-neoplastic agents currently used in the cancer treatment arena.

The production of bioactive peptide hormones by particular cells and tissues is a tightly regulated phenomenon. Well-recognized controls of transcriptional events only affect the first steps leading to prohormone synthesis: posttranslational processing mechanisms also provide a number of sites for regulation of the production of specific hormones. It is our hypothesis that control of posttranslational processing in neoplastic endocrine cells, through inhibition of posttranslational processing enzymes, could allow alterations in the production of biologically active autocrine peptides and control of the biology of the tumor.

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Biomarker Intermediate Endpoints and Cancer Prevention[1]

Frank L. Meyskens, Jr.[2,3]

ABSTRACT—Detection of cancer is the definitive endpoint in the conduct of chemoprevention trials. There are, however, several reasons why cancer as the endpoint may not be feasible or ethical: 1) the usage of patients with easily followable preneoplasias may preclude the development of cancer, and 2) the time to a cancer event may be long or the incidence uncommon, even in individuals at high risk. Two major types of biomarker intermediate endpoints should be considered: 1) those that identify individuals at high risk and 2) those that serve as a surrogate for cancer. Various epidemiologic features, including family history, have been used to estimate relative risk. This approach, however, only slightly decreases the size of populations needed for chemoprevention trials and only little addresses the question of individual risk. Advances in understanding the genetic basis for cancer will lead to the development of probes that will help assess risk for many cancers. Innumerable biomarker intermediate endpoints can be identified as associated with cancer formation, including genetic, epigenetic, and histologic features. The challenge is not in identifying potential biomarker intermediate endpoints but in showing that they are relevant. Carcinogenesis has been shown to be carcinogen, inhibitor, dose, tissue, and species specific; it is likely that relevant biomarker intermediate endpoints will need to be identified, studied, and verified in human models. The upper aerodigestive system should be a rich source for biomarker intermediate endpoint studies, as tissue is readily available, the carcinogenic process can be monitored, and there are currently available reasonable compounds to use in biomarker intermediate endpoint modulation and chemoprevention trials. This human model should be used for extensive in vitro characterization of the biomarker intermediate endpoint/chemoprevention concept. [J Natl Cancer Inst Monogr 13:177-181, 1992]

DIFFERENCES BETWEEN PREVENTION AND TREATMENT TRIALS

The definitive endpoint for assessment of efficacy in cancer treatment trials is survival of the patient (or a group of similar patients) measured from the time of onset of disease to the death of the individual. There is, therefore, a clearly measurable amount of time from the beginning of the disease process to the end. Additionally, a large number of clinical, radiographic, and biochemical measurements are available to accurately serve as estimators of tumor presence and amount. These are used to determine the status of the disease as well as the response of the malignancy to intervention, and response and survival endpoints can be simply identified.

The current situation with the assessment of endpoints in cancer prevention trials is considerably more complex, and the evaluation of endpoints can be divided into five categories: relative risk assessments, intermediate markers, response, incidence, and survival. The major emphasis of this paper will be on the first two categories: relative risk assessment and intermediate markers. We have discussed the issue of response (of preneoplasias) elsewhere (*1–3*) and incidence and survival estimates have been discussed extensively by others.

TYPES OF BIOLOGIC MARKERS

The definitive endpoint for cancer prevention trials is generally regarded as incidence of cancer in a defined population, although properly speaking, the definitive endpoint probably should be change in survival of the patient (or group of patients). Clearly, if the field of chemoprevention is to advance more rapidly, measurements that can accurately predict at an early stage the definitive outcome (appearance of measurable malignancy—preneoplasia or neoplasia) will be extremely valuable. Generally, such measurements or markers are called biomarker intermediate endpoints and are lumped in one group. However, we propose that at least two broad areas should be considered: relative risk assessments and progression or intermediate markers. In thinking about the topic of cancer prevention, it is useful to keep in mind the biologic processes at work in cancer formation (*1*). These are summarized in Fig. 1, and the general types of biomarker intermediate endpoints are listed in Table 1.

Briefly, the important biologic parameters that contribute to or are associated with cancer formation can be divided into two broad categories: relative risk assessments and intermediate or progression markers. Among the former are the intrinsic genetic milieu, rate and degree of activation and/or metabolism of the carcinogen, damage to DNA, and appearance of second malignancies. Direct measurements of progression (intermediate markers) include ongoing and sequential expression of abnor-

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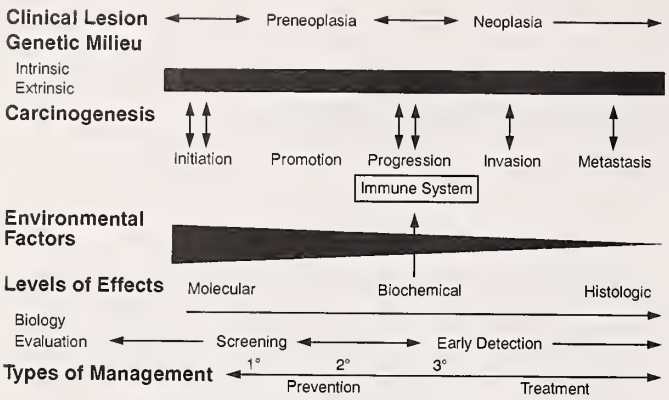

Fig. 1. Relationship of the biology of cancer formation and chemoprevention.

mal growth at the molecular, biochemical, and histologic levels. Indirect measurements of progression include changes in the immunologic status of the host. Interaction of the host with intrinsic (e.g., hormones) or extrinsic (e.g., dietary factors, chemoprevention agents) modifiers of this process also need to be recognized.

GENETIC MARKERS

The intrinsic genetic makeup of the individual may well provide a baseline relative risk factor for cancer formation on which environmental and other factors interact. Several major lines of evidence suggest that these perceptions are true: the general observation that genes play a crucial role in activating (proto-oncogenes) or suppressing (tumor suppressor genes) cellular functions, the characterization of the molecular lesion in several forms of childhood cancers, and the heritable nature of adult cancers in many families.

Table 1. Biomarker intermediate endpoints

Parameter	Marker
Risk assessment	
Intrinsic genetic milieu	Genetic risk
Metabolism of carcinogens	Carcinogen metabolism
Damage to DNA	Molecular (DNA adducts)
Second malignancies	micronuclei changes
Intermediate (progression) markers	
DIRECT	
Progressive changes	
• Molecular	Gene expression
• Biochemical	
• Proliferation	Altered DNA synthesis
• Differentiation	Progression markers
• Histologic	Preneoplasia
INDIRECT	
Immunologic	Cellular or hormonal immunity
Chemopreventive agent	
• Intrinsic	Hormone measurement
• Extrinsic	Levels of compound (serum or tissue)

Discovery and characterization of gene deletions in chromosomes 11 and 13 that lead, respectively, to the development of retinoblastoma and Wilm's tumor has unequivocally established the importance and contribution of genetic alteration to cancer risk (*4–6*). Considerable evidence has accumulated that the retinoblastoma or Rb gene is involved in adult cancers as well, although at later stages of malignant formation. These and other findings have spurred considerably the interest in both the molecular biology and the genetic epidemiology of adult human tumors.

The impressive work of Fearon and Vogelstein has demonstrated that a series of gene deletions and activations cumulatively occur during the development of colon cancer (*7*). In patients with hereditary colon cancer, a specific gene deletion in chromosome 5 has been identified as a constitutive marker of the disease. Abnormalities in this gene also occur in the polyps of adults with nonheritable forms of colon cancer. Whether subtle abnormalities of this gene can be identified constitutively in normal cells of individuals has not yet been established. If a genetic marker of risk for any adult cancer can clearly be identified in the genes of cells of unaffected individuals, this will be an important and critical contribution in many ways. Lynch and his colleagues have been in the forefront in the study of genetic epidemiology of cancer families for quite some time, and strong evidence for a familial or hereditary contribution has been established for some breast, ovarian, and colon cancers (*8*). The delineation of the relative contribution of the intrinsic genetic makeup (that which makes us individuals), germ line mutations, and acquired mutations to the relative risk for cancer will be an important task in the next decade. Apropos of prevention studies, determination of risk by genetic measurements or identification of families at high risk should be increasingly done in planning chemoprevention trials and will allow markedly more precise identification of individuals at truly high risk and will substantially reduce sample size in trials.

A second major area of genetic susceptibility that needs to be more vigorously explored includes the old but reappreciated area of individual heretogeneity in metabolism of carcinogens. It has been known for quite some time that the interaction between carcinogens and the host is regulated by two essential steps: activation to active metabolites and degradation to inactive metabolites (*9*). Enhancement of the first step contributes to increased risk, whereas stimulation of the second results in decreased risk, and vice versa. The enzymes and the genes giving rise to these proteins have been well characterized. It has long been appreciated—particularly in outbred species—that there is a range of susceptibility to the effects of carcinogens and subsequent tumor development. Particularly impressive in humans has been the apparent range of susceptibility to tobacco exposure in terms of subsequent lung cancer development. Recently, a series of papers has convincingly demonstrated a relationship between the ex-

pression of carcinogen metabolizing genes and lung cancer development (*10,11*). It is likely that further definition of carcinogen metabolizing genes and the relation to cancer risk in specific tissues will be a fruitful area for risk-assessment development. Rapid advances in our understanding of intrinsic genetic risk for cancer as well as risk based on metabolism of carcinogens should increasingly allow relative risk profiles to be determined and assigned.

Damage to cellular constituents, particularly DNA, is a reasonable marker to consider as an endpoint. The formation and measurement of DNA adducts after acute exposure to various carcinogens has been well studied; however, little is known about the persistence of DNA adducts in carcinogen-exposed tissues (*12*). Although the identification of DNA adducts may be useful in establishing exposure—and therefore potentially a relative risk—effective use of this marker as an "intermediate" indicator of cancer development or a response to a chemopreventive agent seems unlikely. An even more gross estimator of DNA damage has been the assessment of micronuclei chromosomal fragments containing extranuclear DNA. They have been quantitated in various human tissues, and a series of studies has shown high micronuclei frequency to correlate well with cancer risk in smokers and patients with precancerous lesions (*13,14*). However, in chemoprevention trials in patients with oral leukoplakia or esophageal dysplasia receiving beta-carotene plus retinol or zinc plus vitamin A plus riboflavin, respectively, reduction of micronuclei did not translate to favorable alteration of the precancerous lesions (*15–18*). Whether these conflicting results suggest that micronuclei are not a useful intermediate marker is unclear. Micronuclei may well be a good estimator of risk but may not be sufficiently modulatable to be an "intermediate" marker.

A definitive and important marker of risk is the assessment of risk for second malignancies. A remarkable amount of data are available about risk for second malignancy for a large number of tissues and organs (*19,20*). Because the time to development is relatively short, investigation of chemoprevention agents in these groups of patients should be particularly attractive.

The accumulation of intermediate molecular, biochemical, and histologic changes is a prominent feature of postinitiation progression during cancer formation. As the molecular changes of early cancer formation are more precisely defined, the gene products will be more accurately measurable. Determination of the presence or absence of well-defined gene products and their modulation is likely to be an important area for biomarker intermediate endpoint development in the immediate future. Rb, p53, Her2/new, $p21^{c\text{-}ras}$, and other proteins are being explored as *tumor* markers. It is reasonable that their presence be measured in at-risk individuals as well.

NONGENETIC DIRECT MARKERS

A very large number of candidate biochemical markers of tumor progression has been proposed and can be separated into two broad categories: measurements of proliferation and measurements of differentiation. A substantial amount of the interest in proliferative markers has been generated by the work of Lipkin using colon epithelial tissue as the target and changes in in vitro tritiated thymidine labeling content and patterns as the biomarker (*21*). Impressive correlations with disease risk and modulation by chemopreventive agents have been demonstrated. Nevertheless, the particular assay used is quite difficult, and so investigators have turned to other measurements of proliferation such as Ki67 and BudR. Because hyperproliferation has been found in almost all model systems as a frequent, if not necessary, precondition to frank cancer formation, assessment of the parameter of proliferation is likely to serve as an important intermediate biomarker for human studies.

Changes in parameters of differentiation are nearly uniform features of cancer development. With respect to aerodigestive malignancies, this topic has been addressed extensively in this symposium and elsewhere (*22*). Characterization of other tissue systems has been less extensive, except for melanocyte to melanoma progression and transformation (*23*). Clearly, differentiation parameters at the analyte and antigenic levels will serve both as useful markers of risk and as intermediate markers of progression, and modulation of that process by chemopreventive agents can be readily accessed.

The assessment of histologic changes—that is, preneoplasia—should serve both to assign risk and to provide a clearly assessable intermediate marker of biologic change. We have discussed this extensively elsewhere (*1–3*). Briefly, studies of human preneoplasia offer abundant opportunities to understand the biologic features of human cancer. Remarkable progress has been made in our understanding of the etiology of cervix cancer in the past decade by such studies, and substantial progress in our appreciation of melanocyte progression has occurred as well. The assessment of preneoplasia as a marker for prevention trials presents unique opportunities, as several different independent response parameters can be assessed: 1) Regression to a lower degree of preneoplasia, 2) progression to a higher degree of preneoplasia, and 3) status of surrounding "normal" tissue and its relation to the preneoplasia (e.g., field effect).

NONGENETIC INDIRECT MARKERS

The assessment of indirect markers of cancer formation is also worth considering. Particularly important—and neglected—is the status of the immune system. In experimental models the host defense status, at both humoral and cellular levels, has been shown to be critical in the development of early cancer formation and control. Surprisingly few studies on humans exist. A recent study showed a direct relationship between prognosis of cervix cancer and immune status, and an immunologic staging system was proposed (*24*). Studies in other systems are needed.

Determination of effects of the chemoprevention agent itself should also be closely considered. Of course, decrease of the relevant risk or modulation of an intermediate progression endpoint in a favorable fashion is the desired goal. However, correlation of that change with the definitive endpoint (appearance of preneoplasia or neoplasia) may take some time. Other endpoints can include intrinsic effects of the chemoprevention agent, such as consistent reduction of hormone levels or effect by a dietary strategy (e.g., fat reduction) or by a chemoprevention agent (e.g., tamoxifen). Because a large number of cancers are hormone dependent—and many, if not most, are growth-factor dependent—determination of the effect on hormone and growth-factor levels will be increasingly important in the future.

The pharmacology of dietary and chemoprevention agents also presents ample opportunity for marker measurement. The levels of compounds or metabolites themselves can serve as a useful marker of patient or study compliance. The pharmacologic investigation of chemoprevention compounds has become increasingly sophisticated; serum levels are only the first estimate of intake and tissue distribution, and actual effect on biologic endpoints has become increasingly important (*25,26*).

There has been extensive discussion elsewhere about validation of intermediate endpoints (*27,28*); however validation of biomarker intermediate endpoints in humans or populations is a lengthy process. There are innumerable biomarker intermediate endpoints that can be identified as associated with cancer formation, including genetic, epigenetic, and histologic features. The challenge is not in identifying more markers but in showing that they are relevant. Carcinogenesis has been shown to be carcinogen, inhibitor, dose, tissue, and species specific. It is likely that relevant biomarkers will need to be identified, studied, and verified in vivo in human models. The upper aerodigestive system should be a rich source for biomarker intermediate endpoint studies, as tissue is readily available, the carcinogenic process can be monitored, and there are currently available reasonable compounds to use in biomarker intermediate endpoint modulation and chemoprevention trials. This opportunity for advancing our understanding of the biologic basis of chemoprevention by using appropriate human models is considerable; the aerodigestive system of approval represents a particularly attractive one for such studies, and obtained results should enhance our knowledge of the field substantially.

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Rational Targets for the Early Detection of Lung Cancer[1]

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ABSTRACT—The fact that routinely effective treatments for disseminated lung cancer are not available has prompted the search for effective early detection systems. It is important to identify lung cancer while it is still confined to the bronchial epithelium and is potentially curable with local modalities. We have previously reported on an immunologically based assay to identify antigens expressed on shed bronchial epithelial cells. This assay resulted in a statistically significant correlation of immunostaining with the eventual development of lung cancer 2–4 years prior to routine clinical detection. Attempts to further improve this approach require an understanding of the basis for its success. Based on the work of Hakomori and coworkers, this difucosylated Lewis X structure would be a likely marker of carcinogenic transformation of the bronchial epithelium. In fact, an antibody to this structure was useful for sputum immunocytochemistry analysis for early lung cancer detection. Other carbohydrate structures would also be reasonable markers to evaluate for early detection application, based on the known pattern of expression of these structures in fetal, dysplastic, and neoplastic lung tissue. Another antibody used for sputum immunostaining recognizes a 31-kd protein structure; the antibody is not a known member of a likely class of early detection targets. The reported cases of lung cancer missed by the immunostaining approach included principally adenocarcinoma of the lung, suggesting that the addition of a marker(s) of that type of morphologic differentiation should be considered. Markers to dissect the various forms of lung adenocarcinoma are being characterized and are available for evaluation in early detection applications. Alterations in cell surface antigens that occur during the development of cancer parallel underlying genetic mutations in the preneoplastic cell. In lung cancer, some of the target genes undergoing these mutational events have been identified and characterized and include a number of dominant and recessive oncogenes. With determination of the precise chronologic occurrence of these mutational events and correlation with known pathology, these genetic lesions may provide excellent potential targets for early detection assays. Tumor promotion events also provide another class of targets for early detection consideration, including growth factors. The goal of achieving clinically meaningful early lung cancer detection may require complementary analyses with different classes of markers that evaluate different aspects of the field carcinogenesis of the bronchial epithelium. [J Natl Cancer Inst Monogr 13:183-190, 1992]

Despite intensive research efforts, the mortality rate associated with the diagnosis of lung cancer approaches 90% (*1*). This failure rate reflects the absence of consistently effective systemic therapy for this disease. Because a large percentage of the population is composed of past or present smokers, the current national mortality statistics with lung cancer accounting for 34% of male cancer deaths and 21% of female cancer deaths are not expected to improve for the foreseeable future (*1*). Unfortunately, many nations will experience lung cancer statistics similar to or exceeding those in the United States by virtue of the international surge in tobacco consumption over the last three decades. These factors compel a renewed effort to improve early detection of lung cancer.

In considering the progress that has been made in lung cancer research, the greatest strides may have been in the growing understanding of tumor biology. There has been an explosion in our understanding of the processes of cellular transformation, tumor progression, and invasion and metastasis. This knowledge base may provide the foundation for a new approach to lung cancer management. The majority of unsuccessful clinical efforts to date have comprised therapeutic approaches with empirically identified compounds. A more rational orientation is feasible. We will review a series of approaches to lung cancer control, all of which are based on consideration of our knowledge of tumor biology.

ORIGINS OF EARLY DETECTION OF LUNG CANCER

The work of Saccomanno et al provided the impetus for testing the combination of sputum cytology and chest

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x ray for early detection of lung cancer (*2*). Because cure for lung cancer was achievable only with complete surgical resection of a primary tumor, the development of an effective early detection approach was directed at extending the cure rate with surgery by identifying a greater percentage of surgically resectable patients (*3*). The results of that effort have been extensively reviewed (*3,4*), and no improvement of lung cancer-related mortality was observed with the combination early detection approach. This negative trial had a chilling effect on research in this field that still persists. The strategy of early detection to identify lung cancer when the primary tumor is still localized and amenable to a curative local procedure is sound. The difficulty is that conventional sputum cytology and chest x ray are not sensitive enough detection tools to permit this.

In regard to early detection of lung cancer, a number of considerations, which are summarized in Fig. 1, are relevant. The most important concept was demonstrated by DeVita some years ago, based on experiments with tumor cell kinetics (*5*). From the time of the first transformation event that starts a cancer to the time a clinically detectable tumor burden exists ($> 1 \times 10^9$ or roughly 1 cm^3 nodule of tumor), a variable period of time—from 5 to 20 years, involving 25 or more tumor doublings—may have ensued. The rate of tumor doubling during that preclinical period is not known, but it is reasonable to presume that it is variable. For the sake of simplicity in Fig. 1, the rate is shown as increasing constantly through time.

Because a lethal tumor burden may involve only several more tumor doublings, the designation of a 1- to 3-cm^3 tumor nodule (a clinical stage I lesion) as early tumor is

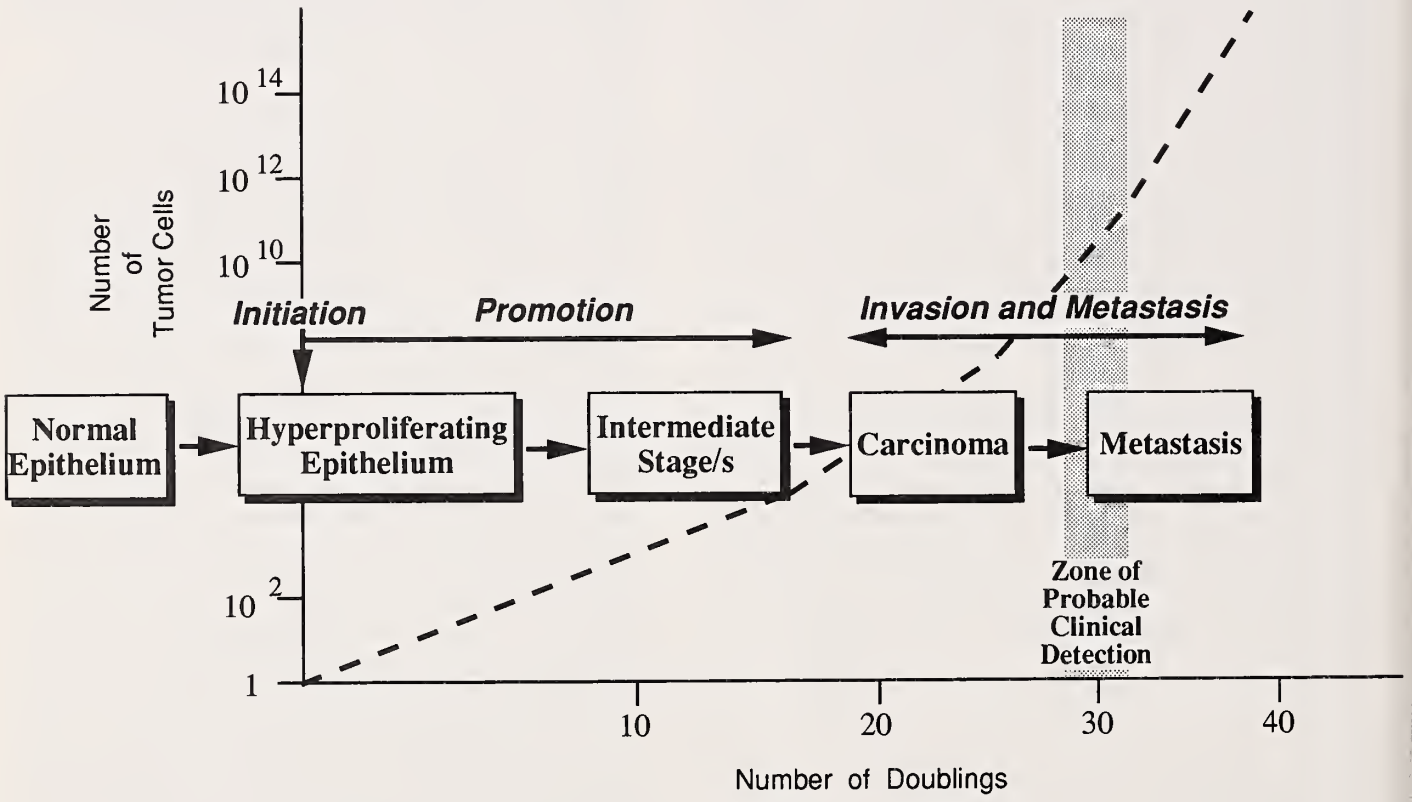

Fig. 1. The physical evolution of epithelial cancer overlaid with a schema of the molecular events in cancer progression. *As indicated by the gray bar,* patients come to clinical detection late in the natural history of the tumor and often with cancer at a time after tumor metastasis has occurred. *Area to the right of the gray bar* represents the principal focus of clinical and basic cancer research to date. The challenge of preventive oncology research is to begin to systematically study the biology and explore the clinical opportunities in approaching early cancer or the process of carcinogenesis itself. The focus of research would then shift to the area on the left side of the gray bar. (Figure modified with the permission of *Oncology.*)

misleading (*6*). This point is reinforced by clinical trials data, which show that the first site of lung cancer relapse occurs in a metastatic location for 75% of patients not cured by the surgical procedure for documented stage I lung cancer (*7*). This means that often distant metastases occur before the best detection tools can identify the existence of lung cancer, even in patients in the most favorable stage.

The combination of chest x ray and sputum cytology screening led to the detection of only 63% of the total new lung cancers in the aforementioned early detection trial (*4*). The remaining 37% of cases were discovered when evaluation of a symptom that led to the discovery of a lung cancer. Combination screening with chest x ray and sputum cytology failed because it was not sensitive enough to pick up a large percentage of cases. In addition, most of the cases picked up by combined screening identified tumors that had already disseminated from the primary site.

Because disseminated lung cancer is amenable only to systemic approaches, and because chemotherapy options for this disease are generally palliative, a functional boundary for effective early detection of lung cancer is defined as indicated in Fig. 1 by the gray bar. To permit routinely curative outcomes with early detection approaches, the early detection tool must identify a stage of lung cancer that is still localized to the primary tumor site (to the left of the gray bar in Fig. 1). The gray bar symbolizes the range of tumor burdens routinely detected with current diagnostic tools. Unfortunately, this also overlaps with the hypothetical zone in which a primary tumor evolves the biologic competence to establish as a metastatic focus in a distant site, as evidenced by the fact that 60% to 70% of patients have disseminated lung cancer at initial diagnosis.

The original strategy proposed by Saccomanno is still correct. Lung cancer universally arises from the bronchial epithelium, and this process can be most closely monitored by analyzing the bronchial epithelial cells shed directly into the sputum (*2,8*). Other currently available radiologic imaging techniques are not thought to be significantly more effective than standard chest x ray to identify new cancers. Any early lung cancer test involving a blood analysis would also be of limited value, since the tumor in question has established access to the systemic circulation and therefore may have already had the opportunity to successfully disseminate. Therefore, lung cancer screening with a blood test as well as with conventional sputum cytology and chest x ray is inadequate based on the high likelihood of detecting lung cancer that has already metastasized and given the lack of effective systemic therapeutics.

The prospect for developing a more successful early detection approach arises from considering the biology of tumor progression to identify targets that lend themselves to clinical application. An example of this comes from the seminal work of Fearon, Vogelstein, and coworkers (*9*) in colon cancer tumor biology. The bottom of Fig. 1 is a simplification of the work of Vogelstein and colleagues in describing a model for the genetic steps in the evolution of a colon cancer. This is a paradigm derived from a systematic effort to describe the mechanistic basis for tumor progression. With maturation of this work will come an appreciation of the molecular signature associated with a particular stage of tumor differentiation. As shown in Fig. 1, there may be a relatively long period from the initial malignant transformation of an epithelial cell to progression of the tumor to the point when it comes to clinical attention. Molecular characterization of a specimen of colon tissue from a subject at high risk for colon cancer could be performed to evaluate the state of differentiation and to discriminate which individuals have experienced molecular changes suggestive of a developing colon cancer. The work of several groups demonstrates that the parallels between colon cancer and lung cancer are very strong (*10–12*), suggesting that the same strategy for lung cancer would be appropriate. Phenotypic manifestation of particular genetic patterns may be more practical to consider for mass screening applications, and so considerations of other classes of markers, including tumor-associated antigens and growth factors, will be considered.

IMMUNOSTAINING ANALYSIS FOR EARLY DETECTION OF LUNG CANCER

To develop an effective early detection approach, we attempted to increase the sensitivity of direct cytologic evaluation of the shed bronchial epithelial cells. This involved immunostaining sputum specimens with tumor-associated monoclonal antibodies using a double antibody bridge technique (*8*). Immunostaining cytospin preparation of sputum presumably worked as an early detection tool, since it identified antigens that were overexpressed on malignant bronchial epithelial cells and permitted the identification of individuals with malignant cells in their sputum. This report was provocative because the analysis involved an archive of serial stored sputum specimens that were acquired in the course of the previously discussed combined chest x ray and conventional sputum cytology study at Johns Hopkins (*3,4*). Long-term clinical follow-up (mean of 80 months) was available on those patients such that reliable determination of who did or did not develop cancer was available. The result of this prospective, nonconcurrent analysis using two monoclonal antibodies for the immunostaining showed that the accuracy of predicting lung cancer status, 20 months prior to the clinical diagnosis of cancer in the patients who developed cancer, was 90% (*8*). The identical analysis was performed on sputum specimens obtained on average 40 months prior to the clinical detection of lung cancer in the subjects who developed lung cancer (with a matched selection of archived material from patients who never developed cancer). This analysis resulted in a 70% accuracy of determining correct lung cancer status in the test subjects, based on immunostaining outcome.

A critical question to ask is whether these results are reproducible. A study has begun to validate this finding independently in a prospective trial using a different pa-

tient population. Pending the result of that trial, it is appropriate to consider the scientific basis for tumor-associated antigens to play a role as early lung cancer markers.

Part of the answer to this question arises from the seminal work of Hakomori, who described the biology of carbohydrate expression in normal, fetal, and malignant tissue (*13,14*). Epithelial tissues characteristically display carbohydrate differentiation antigens on their surface; the most well-known members of this class of molecules are the blood group antigens. The expression of these structures evolves from the fetal state to the mature state. With carcinogenic injury, or in certain cases of severe benign injury, this sequence of antigen display is reversed to the more primitive fetal phenotype. Hakomori and other investigators have identified several patterns of carbohydrate antigen that are more closely associated with neoplasia than with severe benign injury (Table 1) (*13–17*).

In collaboration with the late Dr. Ginsburg, we have identified that one of the two monoclonal antibodies used for the sputum immunostaining, 624H12 (*15*), recognized a difucosylated Lewis X carbohydrate structure, which is of the same family of carbohydrate structure described by Hakomori (*13*). Supporting Hakomori's hypothesis, Itai and colleagues mapped the normal, fetal, and malignant expression of two related carbohydrate structures in the lung and demonstrated directly the concept of reversion of carbohydrate expression for malignant bronchial epithelial cells (*16*). Taken collectively, this work suggests a very central role for carbohydrate antigens as rational markers for early lung cancer detection. Interest in this class of molecules for early detection application is also bolstered by the relative robustness of these structures, which permits rapid clinical analysis with routine immunologic assays. A wide range of well-characterized monoclonal antibodies are already available for this type of application by virtue of the characteristic immunodominance of these structures.

The greatest barrier to validating the utility of this class of early detection tool is the lack of a large repository with serial sputum specimens acquired from an appropriate study population in which clinical follow-up is available. It is only with this type of prospectively acquired panel that the predictive accuracy of an early detection tool can be accessed.

In the past, much attention has been focused on the possibility of serum-based early detection assays; as such, large serum banks were maintained for early detection-marker research. The reasons for the lack of success with this approach may be explained on the basis of the pharmacology of tumor antigens. Lung cancer arises on the bronchial epithelium, and for an indeterminate length of time before the tumor has established a vascular supply, dysplastic cells are shed into the bronchial secretions and cleared into the sputum. The shed neoplastic cells can be recognized on conventional cytomorphologic criteria only rarely (*4,18*). As previously discussed, we used tumor-associated antibody markers that identify potentially neoplastic cells among populations of dysplastic cells due to the overexpression of a particular antigen. If one calculates the number of tumor cells that are required to produce a detectable amount of antigen for a particular marker, then the amount of tumor cells required in the bronchus for detection (as a function of the potential volume of distribution in the airways compartment as recovered in the sputum) is orders of magnitude less than the tumor burden required for antigen detection in the bloodstream.

As demonstrated in Fig. 1, there is a relationship between the age of a tumor and its size. As a corollary, if several orders of magnitude fewer tumor cells are required for an assay from the bronchial compartment compared to the intravascular compartment, then the assay of the bronchial material potentially could be informative years before the intravascular assay. Another issue in comparing tumors localized within the bronchus with disseminated tumors is that tumors that progress to develop the competence for angiogenesis, invasion, and metastasis generally act in a much more aggressive fashion than primary tumors (*19*). On theoretical grounds, including both pharmacology and tumor biology issues, sputum-based early detection would appear far more sensitive than serum-based approaches. Because of the importance of this question, definitive experimental data are required to resolve this issue responsibly.

Table 1. Postulated carbohydrate changes suggestive of cancer

Type of change	Reference
Novel structures (eg, dimeric or trimeric Le^x)	*14, 15*
Restricted structures (eg, incompatible A)	*14*
Overexpression of a common structure (eg, monomeric Le^x)	*14*
1-6 Branched oligosaccharides	*19*

OTHER TUMOR-ASSOCIATED MARKERS FOR EARLY DETECTION OF LUNG CANCER

In the sputum immunostaining report, 26 subjects developed lung cancer, and the antibody to difucosylated Lewis X structure (624H12) detected all five of the small cells and three of the squamous cancer cases (*8*). With some cases of overlap, the remaining antibody (703D4) recognized 20 of the cases from patients who went on to develop cancer. The biology of the antigen recognized by 703D4 is less well worked out than for the difucosylated Lewis X structure. By immunoprecipitation, 703D4 binds to a 31-kd peptide, and this antigen by immunocytochemistry and flow analysis is restricted to the cytoplasmic compartment (*20*). Immunohistochemical localization of this structure by light microscopy in "normal" lung tissue from lung cancer resection specimens is generally restricted to areas of alveolar hyperplasia with only sparse expression in ciliated epithelial cells. This antigen is strongly expressed in a population of hyperplastic type II pneumocytes. This form of bronchial hyperplasia has been

previously suggested to be a premalignant lesion for peripheral lung cancers (*21*). Identifying the peripheral cancers is of enormous importance because these cancers are becoming the dominant form of lung cancer (*22*). Conventional sputum cytologic detection is sensitive only for lung cancers of squamous origin (*4,18*). The sputum immunostaining technique using the two antibodies was able to detect all histologies of cancer but had the greatest error rate with adenocarcinoma and large-cell lung cancer (50% and 50% true positivity, respectively) (*8*).

One approach to improving the accuracy of this technique is to add more antibodies to the staining panel. It might be most efficient to include additional antibodies with known specificities for antigens expressed on adenocarcinomas and large cells. In particular, several new markers to peripheral airway cells have been recently reported (*22,23*). Other normal cell populations of the distal airways may be targets for carcinogenic transformation. Clara cells are very important in the metabolism of many xenobiotic compounds (*24*). The antibody CRL-10 recognizes a specific 11-kd antigen, which is selectively expressed by Clara cells (*25*). Validation of the utility of peripheral airway markers for early detection will require the type of clinicopathologic specimen archive that was previously discussed.

In a recent review we discussed issues relevant to the generic process of early detection screening for lung cancer (*26*). One element of that discussion bears mention here. For the vast potential lung cancer population, a very cost-efficient technique is required. The assay must also be highly accurate and reproducible. For all of these reasons, the conventional endpoint of visual interpretation by a pathologist is a potential limitation. To quantitate the endpoint, the incorporation of a computer-integrated image analysis system into this process is promising (*27*). Further refinement of image analysis techniques will be required to permit a cost-effective, reproducible, and quantitative endpoint determination for population-based screening.

Another challenge in developing lung cancer screening approaches is imposed by the fact that the at-risk population is so large. Strategies to stratify risk groups where the high-risk group becomes the target for the screening would potentially improve the efficiency of the process.

In the proposed validation of the sputum immunostaining for early detection of lung cancer, a population of stage I resected non-small-cell lung cancer patients will be studied. This group has a risk of lung cancer 10 to 100 times higher than a heavy smoker, so the cost basis of the trial is more economical. Care will be required in extrapolating the results of screening approaches validated with very high-risk patients back down to lower risk populations because of the statistical considerations in determining the predictive accuracy of a test.

Potentially, the accuracy of lung cancer screening can also be improved by using more than one type of assay. For instance, in addition to cell surface antigens, growth factor expression or oncogene status may further indicate the presence of an early cancer. Algorithms that include combinations of assays or sequences of assays will need to be validated to improve the precision of population-based screening.

MOLECULAR TARGETS FOR EARLY DETECTION

The application of modern molecular biologic techniques to epithelial cancers has identified genetic lesions in a number of key target genes that are critical for regulating cell growth (*28*). These genes, known as oncogenes, can be classified on the basis of their ability to regulate growth in a positive or negative manner (Table 2). Dominant oncogenes undergo specific activating mutations such as promoter deregulation, sequence substitutions, mRNA stabilization, or amplification, while recessive oncogenes undergo inactivation by deletion or mutation. Data accumulated from many different laboratories studying a variety of epithelial tumors have shown that multiple different genetic events frequently occur during the development of any one of these tumors, but similar patterns of mutations are seen among different epithelial tumors (*29–32*).

Although the precise chronologic order of occurrence of these mutational events remains unknown, it is clear that they underlie the pathologic changes that characterize tumor development. As such, they constitute excellent potential targets for screening assays to identify patients with early preneoplastic lesions. This could be accomplished by examining specific tissues from high-risk patients for mutations in or elevated levels of specific oncogene products.

Table 2. Target genes and their genetic lesions in human tumors

Oncogene	Genetic lesion	Site	Reference
Growth-stimulating ras	Point mutation	Lung(s)	*37*
		Colon	*38*
		Pancreas	*39*
Her-2/neu	Amplification, overexpression	Breast	*35*
		Ovarian	
myc family	Amplification	Lung	*37*
Growth-suppressing p53	Deletion, mutation	Lung	*36*
		Colon	*40*
Rb	Deletion	Lung	*37*

In the lung, the tissue would include bronchoscopic biopsies of dysplastic or metaplastic epithelium or sputum specimens. The candidate target genes include ras, Her-2/neu, myc, and p53 (Table 2).

Activated Kirsten ras genes have been identified in approximately 30% of adenocarcinomas of the lung (*33*). These activating mutations have been well characterized and are found in three specific codons. The mutant protein can be assayed in several different fashions: 1) immunohistochemical staining with antibodies specific for the activated form could be used; 2) polymerase chain reaction (PCR) on genomic DNA with specific amplimers (oligonucleotide primers) could be used to obtain sufficient quantities of the ras alleles to be analyzed directly by nucleic acid sequence, single-strand conformational polymorphisms (PCR-SSCP), or differential oligonucleotide hybridization; and 3) PCR using mutated oligonucleotides as amplimers could be used to detect only the activated (mutated) allele. These various techniques are extremely sensitive (some on a single-cell basis) and could be used in sputum analysis as a potential general screening test (*34*). However, it should be noted that the total number of lung cancer cases identified would be limited, since ras mutations occur in only 10%–15% of lung cancer.

A number of other genetic changes have been noted in lung cancer tissues. Her-2/neu is a tyrosine kinase that has been found to be elevated in some non–small-cell carcinomas of the lung. Immunostaining of high-risk tissue samples could be readily achieved, as specific antibodies are presently available. In fact, immunohistochemical staining for this oncogene has already proved to be of clinical value in breast and ovarian cancer (*35*). The myc family of oncogenes has also been found to be amplified and overexpressed in lung cancer tumors (primarily small-cell lung cancer) (*33*). Immunohistochemical staining or quantitative PCR could be methods of determining the presence and level of expression of all three myc family members. Finally, recent data suggest that p53 is a gene that is frequently deleted or mutated in lung cancer (*36*). The technology exists that can provide for the identification of mutations in this gene, even from small samples of tissue. PCR with the appropriate oligonucleotide exon primers allows amplification of the entire coding sequence of this gene. This coupled with direct nucleic acid sequencing or PCR-SSCP analysis could identify p53 mutations in DNA, even from small numbers of cells. Finally, the use of antisera specific for p53 mutations provides an additional or complementing technique to evaluate the presence and expression of the mutated form of this oncogene.

The above approaches are directed at candidate genes in which genetic lesions are known to occur during the development of lung cancer. More work is required to ascertain whether any of these are critical early events in the development of lung cancer. However, even if they are not, the techniques described could be applied to a wide variety of mutations in any given gene if that gene were found to be important in the early preneoplastic phase of lung cancer. Finally, it should be noted that molecular markers for the screening of lung cancer need not be used alone as a single assay. In fact, this approach may be best as a complement to other screening assays, such as those discussed elsewhere in this article. By using both cell surface markers and molecular targets, one may develop an algorithm of sequential assays that would produce a more sensitive and specific screening approach.

GROWTH FACTOR TARGETS FOR EARLY DETECTION AND POTENTIAL INTERVENTION APPLICATION

As outlined in Fig. 1, the phase of early lung cancer that is most favorable for early detection and successful intervention is the preclinical phase prior to the establishment of metastatic disease. During this time the original transformed cell is clonally expanded on the bronchial epithelial surface. This process is referred to as tumor promotion, and it is accepted that growth factors play a major role during this interval.

Our group has been interested in lung cancer growth factors since the elucidation of gastrin-releasing peptide (GRP) as an autocrine growth factor for small-cell lung cancer (*41*). The self-stimulation of a tumor cell by a peptide provides a plausible mechanism for tumor progression. This possibility is underscored by the report of Willey and coworkers that this peptide can stimulate the growth of short-term cultures of bronchial epithelium, when grown in a defined medium (*42*). Recent experiments by Sunday and coworkers have demonstrated that GRP stimulates the growth and maturation of fetal lung cells (especially type II pneumocytes) (*43*). Aguayo and coworkers have shown that the bronchial lavage of subjects who smoke cigarettes has a significant elevation in the measured level of a GRP-like activity when compared to nonsmokers (*44*). These reports suggest that autocrine growth factors may be expressed during fetal development and with processes leading to neoplasia, and so the biology of autocrine growth factors parallels what was suggested by Hakomori for carbohydrate antigens in regards to oncofetal expression (*13*). Growth factors that are critical in organogenesis are generally tightly regulated, such that their expression declines with maturation of the individual. In the course of chronic injury mediated by a carcinogen, such as tobacco, these factors become reexpressed. A conservative speculation is that these factors may play a role in lung repair and more specifically lung carcinogenesis, as summarized in Table 3. Conceptually, one could term these factors neogrowth factors, as Hakomori has designated the oncofetal carbohydrate antigens as neoantigens.

An anti-GRP monoclonal antibody that neutralizes the mitogenic effect of this factor has been used in a clinical trial with advanced cancer patients (*45*). This clinical trial has been remarkably free of side effects but without significant antitumor effect. This situation parallels the clinical status of 13-cis-retinoic acid. Retinoids have a reproducible effect on cancer cells by redirecting the cellular machinery to induce terminal differentiation. This effect

Table 3. Basis for considering GRP as a lung cancer promoter

	Reference
1. Acts as autocrine growth factor for small-cell lung cancer	*41*
2. Stimulates growth of normal adult bronchial epithelium	*42*
3. Gene expression in mouse lung coincides with peak GRP effect	*43*
4. Stimulates growth and maturation of fetal type II pneumocytes	*43*
5. Increases levels found in bronchial lavage of smokers	*44*

was not clinically significant for advanced cancer patients, but when retinoids were administered for a year to surgically treated patients with head and neck cancer, the result was significant suppression of new primary aerodigestive cancers (*46*). These observations taken together led to a hypothesis that growth factors such as GRP are an attractive target for intervention approaches (*6*). If growth factors drive tumor progression, the identification and quantitation of relevant factors may also provide important indications regarding the presence of such promotion dynamics in the airways of an individual.

Other biologic markers, the rate of cellular proliferation, activation of neuroendocrine machinery (such as elevated levels of the enzyme PAM, which is involved with peptide processing), or DNA ploidy status are processes that are known to be perturbed in tumor progression and may be informative as early detection tools (*18*). These markers may actually define mechanisms involved in the cancer field that are central to the development of a pulmonary cancer (Table 4). As with the sputum markers the barrier to determining the utility of such tools is the lack of relevant specimen archives in which clinical follow-up is available. To validate new markers or to conduct the research required to assemble the panels of markers or algorithms for markers as discussed earlier, a major effort to develop serial tissue archives from patients with long-term clinical follow-up will be essential.

CONCLUSION

Given the current limited success for treating patients with lung cancer, a thoughtful reconsideration of lung cancer management strategies is appropriate. The intuitive appeal of a biologically oriented early detection approach provides a compelling focus for lung cancer clinical research. Because of the clinical consequences of diagnostic misclassifications, analytic rigor is imperative. Rational approaches to early detection of lung cancer may cross-fertilize rational approaches to lung cancer intervention as well. With the successful development of better tools for early detection, new classes of lung cancer intervention tools will be required to specifically address the small tumor burdens that would be expected. Simultaneously, the need for long-term follow-up and the expectation of subsequent additional new lung cancers also provide a challenge for the development of useful new intervention approaches. Higher priority has to be placed on the systematic evaluation of patients at high risk for cancer, with archival storage of clinical specimens. The rate of progress in this field depends on such strategic investments.

Table 4. Markers of field cancerization

Change in carbohydrate composition
Expression of neoenzymes (1-3)-L-fucosyltransferase
Change in growth factor composition
Up-regulation of neuroendocrine machinery (PAM activity)
Up-regulation of proliferation markers

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Association Between Histological Type and Neuroendocrine Differentiation on Drug Sensitivity of Lung Cancer Cell Lines

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ABSTRACT—Because most small-cell lung cancers (SCLC) are initially chemosensitive and express neuroendocrine (NE) cell markers and most non-SCLC tumors (NSCLC) are chemoresistant and do not express NE cell markers, we investigated the association between morphological type and NE cell differentiation with in vitro chemosensitivity. We tested a panel of 55 lung cancer cell lines established from previously untreated patients. These were tested against five cytotoxic drugs commonly used in the therapy of lung cancer, using the MTT assay. For comparative purposes, we also tested cell lines established from previously treated patients with SCLC and from colorectal tumors. The logarithms of the IC_{50} values of all of the cell lines were normally distributed, permitting the use of Student's *t*-test for assessment of differences. In general, the in vitro sensitivities of SCLC, NSCLC, and colorectal cell lines mirrored the clinical experience with these tumor types. Cell lines started from previously treated patients with SCLC were more resistant than those from previously untreated patients who responded to initial therapy. For all of the cell lines, the sensitivities to the five drugs tested were highly significantly correlated with each other. Thus, for comparative purposes, each group could be assigned an average standardized mean rank. About 15% of NSCLC tumors express multiple neuroendocrine (NE) cell markers and 4 of 5 lines from these NSCLC-NE tumors were relatively chemosensitive, similar to SCLC lines and significantly different from other NSCLC lines. Other NE cell lines tested included bronchial carcinoids and cell lines from small-cell carcinomas arising in extra-pulmonary locations (ExPuSC). While all four carcinoid cell lines were uniformly chemoresistant in vitro, ExPuSC were heterogeneous, with four of six lines being relatively chemosensitive. These findings mirror clinical experience. We concluded that 1) all lung cancer lines fall into broadly chemoresistant or chemosensitive categories; 2) SCLC lines are more chemosensitive than NSCLC or carcinoid lines; 3) NE cell differentiation may delineate a relatively chemoresponsive subset of NSCLC; 4) cell lines are suitable models for investigating the mechanisms of drug resistance in lung cancer. [J Natl Cancer Inst Monogr 13:191–196, 1992]

Lung cancer is the most common fatal cancer in the United States and in many parts of the world, and nearly 90% of patients will eventually die from the disease (*1*). For a number of clinical, biologic, and therapeutic reasons, lung cancers are frequently divided into two major types: small-cell lung cancer (SCLC), accounting for about 25% of lung cancers, and non–small-cell lung cancer (NSCLC), comprising the other histologic types. The major cause of treatment failure in all lung cancers is drug resistance. While most SCLC tumors are initially responsive to cytotoxic therapy, they usually recur, at which time they are usually resistant to further chemotherapy and even to drugs that had not been administered to the patient (acquired resistance). By contrast, most NSCLC tumors are resistant to chemotherapy at the time of diagnosis (inherent or de novo resistance). Despite these general patterns, approximately 20% of SCLC tumors are initially resistant to chemotherapy, and some NSCLC tumors are initially responsive to therapy (*1*).

SCLC is a neuroendocrine (NE) tumor and expresses all the markers associated with this type of differentiation, including specific peptide production and the general NE markers L-dopa decarboxylase, chromogranin A, synaptophysin, and the presence of cytoplasmic dense core granules (*2–4*). Carcinoid tumors, a relatively rare form of lung cancer, are also NE tumors and are somewhat better differentiated than SCLC (*4*). However, in contrast to SCLC, carcinoids are very resistant to cytotoxic therapy. While most NSCLC tumors lack NE cell markers, about 15% express the entire program of NE cell differentiation (*2*). Finally, tumors morphologically resembling SCLC occur in a wide variety of extra-pulmonary sites including

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head and neck, thymus, gastrointestinal and genitourinary tracts, and uterus and uterine cervix (*5*). Although some of these are poorly differentiated examples of other tumor types (especially squamous cell), about half of extrapulmonary small-cell cancers (ExPuSC) express NE cell markers (*5*). At least some ExPuSC tumors have been reported to be chemosensitive (*6*). The estimated incidences of the various NE tumors of the lung and of ExPuSC tumors are reported in Table 1.

While the major predictor of initial chemosensitivity in lung cancer is histological type (SCLC versus NSCLC), other factors may influence outcome. In particular, we were interested in the effects of NE cell differentiation on the in vitro chemosensitivity of lung cancer cell lines. To address these questions, we utilized an available resource, the largest and most comprehensive collection of lung cancer cell lines (*7–10*). These cell lines represent all the major histological types and NE cell phenotypes, including ExPuSC lines. In this report we validate the concept that cell lines are suitable, clinically relevant models for the study of lung cancer, and we compare and contrast the relative in vitro chemosensitivity patterns of the various types of lung cancer described previously.

MATERIALS AND METHODS

Cell Lines and Culture

Methods for establishment, characterization, and maintenance of cell lines have been described previously (*8*). The cell lines were established in our laboratory from tumor specimens obtained during routine diagnostic or therapeutic procedures. The histologic diagnoses were reviewed in most cases by two pathologists experienced in lung cancer (A.F.G. and R.I.L.). A representative panel of SCLC and NSCLC cell lines established from previously untreated patients was selected. All available examples of bronchial carcinoid, ExPuSC, and NSCLC lines expressing NE cell markers (NSCLC-NE) were included. The panel was composed of 19 SCLC, 26 NSCLC, including five NSCLC-NE, four carcinoids, and six ExPuSC cell lines. For comparative purposes, we included cell lines established from colorectal cancers (n = 11) (*11*) and SCLC lines from previously treated patients (n = 20).

Drug Sensitivity Testing

In vitro drug sensitivity testing (DST) was performed using the MTT tetrazolium dye colorimetric assay as described in the literature (*11–13*) and further modified by us (*14*). The panel of 55 cell lines was tested over a period of approximately 18 months. The assay was standardized for individual cell lines by predetermining the optimal seeding densities. Cells were disaggregated, resuspended in the appropriate growth medium, and seeded into 96-well plates in a volume of 180 μL. After approximately 16 hours (to allow cells to recover from disaggregation), 20 μL of drug solution or saline were added to test and control wells, respectively. After exposure to drugs for 4 days, the remaining steps of the assay were described by Tsai et al (*14*). The results, reported as IC_{50} values, were the means of three or more independently performed assays (each assay was performed in octareplicate). The IC_{50} values were defined as the drug concentration required to inhibit cell growth by 50%. The in vitro sensitivities of the cell lines to five drugs commonly used in the treatment of lung cancer were determined. The drugs included etoposide (VP16), cisplatin (PLA), doxorubicin (ADR), melphalan (CYT) (used as an in vitro substitute for cyclophosphamide), and carmustine (BCNU). Personnel performing DST were blinded to clinical data.

Table 1. Estimated annual incidences of neuroendocrine tumors in the United States*

Tumor type	Incidence	Ratio:SCLC
SCLC	37,500	1:1
NSCLC-NE	13,550	1:3
Carcinoid	2,250	1:17
ExPuSC	1,500	1:25

*Estimated from published data (*5,26*)

Determination of the NE Cell Phenotype

Expression of the general NE cell markers L-dopa decarboxylase, cytoplasmic dense core granules, chromogranin A, and synaptophysin were determined as previously described (*2,3,7*). Cell lines categorized as having the NE cell phenotype expressed two or more of these markers.

Statistical Methods

The Kolmogorov-Smirnov test was used to determine whether the logarithms of the IC_{50} values were consistent with a normal distribution. The Wilcoxon rank sum test and Student's *t*-test with Satterthwaite's adjustment (*15*) were used to test for differences in the mean ranks of the cell lines tested. Pearson product-moment correlations were used to determine correlations between drugs.

RESULTS

Correlations of Drug Sensitivities

The IC_{50} values for the five drugs tested were determined for 55 lung cancer cell lines (19 SCLC, 26 NSCLC, including five NSCLC-NE, four carcinoids, and six ExPuSC). For two drugs, etoposide and cisplatin, data from an additional 11 SCLC lines were available and were utilized for comparisons where mentioned. We had previously noted that the IC_{50} values of etoposide and cisplatin were closely correlated in cell lines initiated from untreated patients with SCLC (*14*). We extended these results to the different lung cancer types and for the five drugs tested (Table 2). For the 55 cell lines tested, all the tested drugs were significantly correlated with each other. When SCLC and NSCLC lines were analyzed separately,

Table 2. In vitro drug sensitivity testing of lung cancer cell lines: correlation of drug sensitivities*

	Correlation			
Drug	PLA	ADR	CYT	BCNU
VP16	0.70 (<0.0001)	0.74 (0.0001)	0.49 (0.0004)	0.41 (0.0035)
PLA		0.72 (0.0001)	0.70 (0.0001)	0.48 (0.0005)
ADR			0.59 (0.0001)	0.38 (0.007)
CYT				0.54 (0.0001)

*Fifty-five cell lines representing all lung cancer types discussed were tested, and the IC_{50} concentrations of the five drugs tested were determined. The values in the table represent the standard Pearson correlation coefficients (*27*). Figures in parentheses are the P_2 values.

the Pearson correlation coefficients within each of these tumor types were similar to those for the entire group of lung cancer lines. Because the values for individual drugs were tightly correlated, a mean rank for all five drugs could be assigned to each individual cell line and different cell types could be assigned an average standardized mean rank, with a potential range from 0.01 (the most sensitive) to 1.00 (the most resistant).

Comparison of DST Patterns of SCLC, NSCLC, and Colorectal Cell Lines

Before the DST patterns of the different major groups could be compared, we had to determine whether the distribution of the IC_{50} values of the cell lines were homogeneous. The Kolmogorov-Smirnov test indicated that the logarithms of the IC_{50} values of the SCLC and NSCLC groups were consistent with a normal distribution for all five drugs tested. Therefore, differences between them could be assessed using Student's *t*-test. Fig. 1 illustrates the normalized curves of the values for etoposide and cisplatin. When the standardized mean ranks for all five drugs were compared, the differences between SCLC and NSCLC (P_2 = .0006) and SCLC and colorectal cancer (P_2 = .0001) were highly significant. In addition, a comparison of the mean values for the individual five drugs were all highly significant for these sets of comparisons. When NSCLC and colorectal lines were compared, the differences for etoposide, doxorubicin, and melphalan were significant (P_2 values of .007–.0001) but not for cisplatin or carmustine. As a result, the differences between the mean ranks of these two groups were of borderline significance (P_2 = .057).

Comparison of the DST Patterns of SCLC Lines from Untreated and Previously Treated Patients.

Patients with SCLC who have relapsed after chemotherapy induced remission are usually resistant to further drug therapy. We compared the DST patterns to etoposide and cisplatin of 30 cell lines established from previously

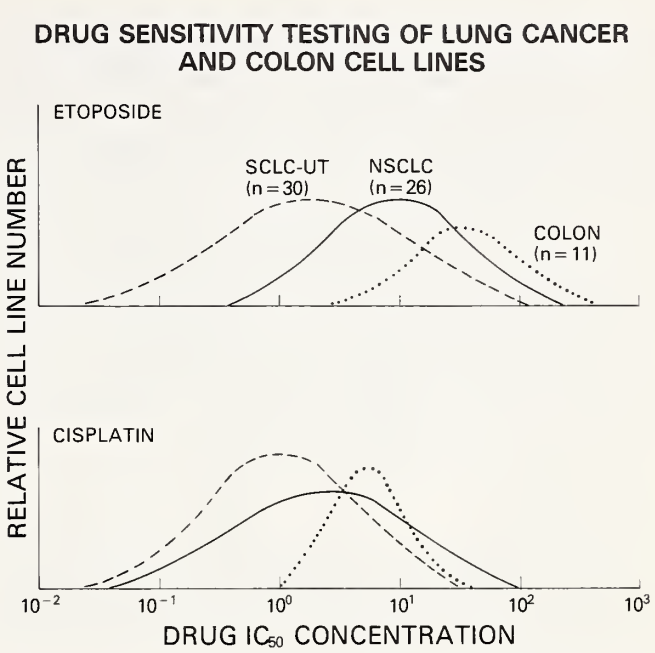

Fig. 1. Normalized curves for IC_{50} values of etoposide and cisplatin having the same means and standardized deviations as the data comparing SCLC, NSCLC, and colorectal carcinoma cell lines. Data for these two drugs were available from 30 untreated SCLC lines and were used here. Data on all five drugs were available on a subset of 19 lines, and they were used for all the statistical analyses presented in the text.

untreated (UT) with those of 20 lines from previously treated (PT) patients. These differences were not significant. We have previously demonstrated that approximately 20% of patients receiving etoposide/cisplatin as initial therapy are non-responders. IC_{50} values for cell lines from responder and non-responder patients are highly significantly different (*14*). When the cell lines from non-responder patients (n = 5) and from those that were not evaluable (n = 4) were excluded, the IC_{50} values for the remaining 21 UT patients were significantly different from the PT group for etoposide (P_2 = .0045) and of borderline significance for cisplatin (P_2 = .056).

Expression of NE Cell Markers in Lung Cancer Lines

An examination of our lung cancer cell line panel for expression of the NE cell phenotype indicated that all of the SCLC, carcinoid, and ExPuSC lines expressed two or more markers. An examination of all 32 NSCLC lines available in our laboratory at the start of these studies indicated that five (16%) expressed the NE phenotype. All five of the NSCLC-NE lines and 21 of the remaining 27 NSCLC lines were selected for DST studies. All SCLC, carcinoid, and ExPuSC lines expressed NE markers.

Influence of NE Differentiation on In Vitro Chemosensitivity

To determine the effect of NE differentiation on in vitro chemosensitivity, we separated the NSCLC-NE group

from the remaining NSCLC lines. As demonstrated in Table 3 and Fig. 2, differences between SCLC and (remaining) NSCLC or carcinoid were major (P_2 = .0001 and .0002, respectively). Of particular interest, the NSCLC-NE group was similar to the SCLC (P_2 = .8) but significantly different from the remaining NSCLC lines (P_2 = .01). Values for four of five of the NSCLC-NE lines were similar to those for SCLC, while one was relatively resistant. All the carcinoid lines were highly resistant to all drugs tested, as indicated by their high mean rank and small standard deviation. The only inconsistent group was the ExPuSC lines, with four relatively sensitive lines and two relatively resistant lines (Fig. 2). Their mean rank was not significantly different from SCLC or NSCLC (P_2 values of .68 and .06, respectively).

DISCUSSION

One of the major challenges in interpreting DST data is determining their clinical relevance. We have previously demonstrated that DST of short- and medium-term SCLC cultures using a dye exclusion test predicted for patient response (*16*). In a more recent study, we extended these findings to long-term SCLC cultures (mean culture time of 29 months) using the MTT tetrazolium dye assay (*14*). In the present report, we demonstrate that the DST results of testing a relatively large panel of cell lines from previously untreated patients indicate significant differences between SCLC and NSCLC or colorectal lines. In addition, there were highly significant differences for etoposide when UT lines from responding patients were compared to lines from PT patients. All of these findings confirm the clinical relevance of our in vitro assays.

Table 3. Average standardized mean rank of lung cancer cell line types*

Tumor type	*n*	Average mean rank	SD
SCLC-UT	19	0.32	0.22
NSCLC	21	0.65	0.19
NSCLC-NE	5	0.30	0.28
Carcinoid	4	0.80	0.05
ExPuSC	6	0.42	0.29

*For each cell line tested, the average rank for the five drugs tested was determined, and the mean standardized rank for each cell line type determined as described in the text. Chemoresistant cell types have a higher average mean rank than chemosensitive types.

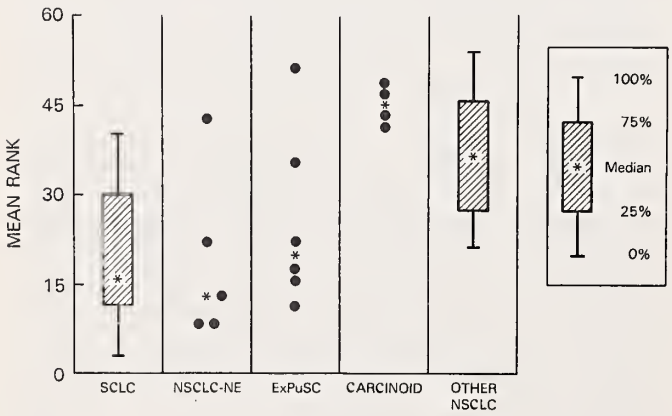

Fig. 2. Mean ranks for lung cancer cell lines for the five drugs tested. Data for SCLC and NSCLC lines lacking NE markers are displayed in box plots. For NSCLC-NE, ExPuSC, and carcinoid cell lines, the individual cell line data are displayed.

In all forms of lung cancer all five drugs tested were highly correlated with each other. These observations confirm and extend those by Carmichael et al (*17*). They indicate that, in general, all lung cancers fall into broad chemosensitive or chemoresistant patterns, and they confirm similar clinical observations. These observations confirm that in vitro tests may be useful for predicting clinical response in lung cancer (*14,16*) but indicate that the tests will be of limited use in the selection of individualized chemotherapy. The mechanism by which some lung cancers become resistant to many therapeutic drugs, including those that they have not previously been exposed to, remains unknown. We have previously demonstrated that expression of the MDR1 gene product is not related to drug resistance in lung cancer tumors and cell lines (*18*). Because no single known mechanism can explain all of the clinical and laboratory findings, it is believed that drug resistance in lung cancer is either due to some novel, as yet unknown, mechanism or is due to some combination of methods acting in concert.

One of the most intriguing aspects of our studies is the possible relationship of NE cell differentiation and drug resistance of lung cancer cell lines. As demonstrated in this report, NSCLC tumors expressing NE markers are as chemosensitive as SCLC lines, while NSCLC lines lacking NE markers are relatively chemoresistant. Because NSCLC-NE tumors constitute a relatively large absolute number of tumors (over 13 000 cases per year in the United States), they may represent an important subset of NSCLC tumors that have a better initial response to therapy and a better prognosis than NSCLC tumors lacking these markers. Our findings (*19*) and those of Graziano et al (*20*) are in keeping with this hypothesis. Further suggestive evidence that NSCLC-NE tumors represent a distinct clinico-pathological subgroup has been provided by other reports (*4,21–23*). Paradoxically, the relatively chemosensitive NSCLC-NE cell lines express relatively high levels of the MDR1 gene (*18,24*). Hainsworth and coworkers have identified a group of poorly differentiated neuroendocrine tumors of unknown origin that are relatively chemosensitive (*25*). Because many of these are located in the thorax, the possibility that some of them represent NSCLC-NE tumors must be considered.

Our findings also confirm the clinical observations that bronchial carcinoids are highly chemoresistant tumors. All four carcinoid cell lines tested were uniformly resistant to all drugs tested. The ExPuSC lines were, in general rela-

tively chemosensitive, but two lines were relatively resistant, suggesting that these tumors, which arise at multiple different sites, may represent a heterogeneous group. At least some ExPuSC tumors have been shown to be chemosensitive (*5,6*). Thus in SCLC and NSCLC, NE marker expression is correlated with the NE cell phenotype, but not in carcinoids and ExPuSC tumors.

The most important single factor in predicting the chemosensitivity of a lung tumor is the histologic type. Thus most untreated SCLC tumors are relatively chemosensitive, while most NSCLC and bronchial carcinoid tumors are relatively resistant. However, an important fraction of untreated SCLC tumors is relatively chemoresistant and will not respond to initial therapy. The mechanism for this chemoresistance is not known. In contrast, an important subgroup of NSCLC tumors that express NE cell markers are as chemosensitive as SCLC lines, and they represent a distinct clinico-biologic subgroup. Our studies also clearly demonstrate the relevance and usefulness of continuous cell lines for studying mechanisms of drug resistance in lung cancer and for exploring newer therapeutic methods for overcoming such resistance.

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Combination Immunotherapy for Advanced Lung Cancer[1]

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ABSTRACT—Therapeutic strategies using cytokines have not been extensively tested in patients with advanced non-small-cell lung cancer (NSCLC). We developed a basic laboratory and clinical research program to investigate the effective immunotherapeutic manipulations for activating the endogenous immune system with biological agents. Our efforts using low-dose interleukin 2 in conjunction with other biologic response modifiers for the treatment of NSCLC are summarized here. [J Natl Cancer Inst Monogr 13:197–201, 1992]

Lung cancer leads all other cancers in the percentage increase of cancer-related deaths. Surgical resection remains the primary and most effective modality for early disease. The addition of radiotherapy and chemotherapy results in only marginally significant prolongation of survival. Smoking cessation programs will ultimately decrease lung cancer mortality; however, their effect will not be immediate, and a high mortality rate will continue into the 21st century. Because current therapeutic modalities have limited usefulness as adjuvants in early disease and in advanced disease, attention has turned toward exploring other treatment options.

Immunotherapy has been suggested as a fourth treatment modality for patients with cancer. Immunotherapy trials in lung cancer began 30 years ago. Initial trials in patients with advanced disease used both nonspecific and specific active therapy. Little antitumor activity was seen in these early studies compared with results from surgery, chemotherapy, or radiation. When interleukin 2 (IL-2) was observed to cause tumor regression in melanoma and renal cell cancer (*1*), our interest in immunotherapy for non-small-cell lung cancer (NSCLC) was renewed.

In an attempt to develop effective manipulations of the endogenous immune system, other biologic agents were investigated that might act synergistically with IL-2 in the induction of lymphokine-activated killer (LAK) activity. Recent evidence in vitro shows tumor necrosis factor-α (TNF) is one such agent that may function as an important mediator in the immunologic cascade of lymphocyte activation. Carswell and colleagues (*2*) orginally described TNF as the possible mediator causing hemorrhagic necrosis in certain transplanted tumors in mice. Later studies (*3–5*) showed that TNF is cytotoxic and cytostatic for various cell lines in vitro. However, in vitro, lower concentrations of TNF appear to have an immunomodulatory role for induction of cytotoxic lymphocytes rather than a direct cytotoxic effect (*6,7*). This mechanism may involve stimulation of lymphocyte proliferation (*8*) or enhancement of high-affinity IL-2 receptor expression on activated T cells (*9*). The use of TNF in Phase I studies in advanced cancers as a cytotoxic agent (*10–13*) led to considerable toxicities, expecially at the higher doses (150 $\mu g/m^2/d$). There were no responses in 86 patients, who included a total of eight patients with advanced NSCLC.

Most of the clinical trials have used IL-2 alone or with LAK cell infusion. In recent clinical trials (*1,14*), this method evoked responses in patients with melanoma, renal cell carcinoma, colon carcinoma, and non-Hodgkin's lymphoma. A small number of lung cancer patients were enrolled in these studies, but no responses were observed (*1,15*). However, stabilization of progressive disease in patients with NSCLC has been observed using a combination of IL-2 plus beta ser 17 interferon (*16*).

Although effective in tumor systems other than lung carcinoma, administration of high doses of IL-2 with or without the adoptive transfer of LAK cells or tumor-infiltrating lymphocytes (TIL) is associated with toxicities related to the increase in capillary permeability. These systemic toxicities and the limited number of responses are major barriers to successful immunotherapy for other tumor types. We focused our laboratory research efforts on using combinations of biologic agents that have low levels of antitumor activity as individual agents, but which may

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act synergistically to augment host antitumor immune responses.

PRECLINICAL STUDIES

Our initial studies showed that the addition of TNF to cultures with suboptimal doses of IL-2 can activate cytotoxic lymphocytes to levels greater than those obtained with higher concentrations of IL-2 alone (*17*). When tested against fresh primary NSCLC cell lines and fresh tissue, induction of cytolytic activity with IL-2 plus TNF was enhanced over IL-2 alone against all cell lines and in 32 of 33 tumor specimens with a median increase of fourfold (range, 0.7–16.0) (*8*). This synergistic activation with IL-2 and TNF used in combination was supported by subsequent studies in vivo (*18–20*).

Another method for induction of cytotoxic lymphocytes may involve stimulation of the invariant region of the CD3 determinant leading to a variety of T-cell functions (*21–24*). High doses of the anti-CD3 monoclonal antibody inhibit antigen-directed cytotoxic activity, as shown by the clinical use of OKT3 for suppression of graft rejection (*25*). However, at 100-fold lower doses, anti-CD3 has mitogenic properties for the T-cell population in vitro (*21–23, 26–28*). Although anti-CD3, IL-2, and TNF have low levels of antitumor efficacy as individual agents, a combination of the three agents could possibly demonstrate increased activity in vivo. This hypothesis is based on the observation that immunomodulation involves several events leading to the production of one or more cytokines that act to amplify the immune response. We reported the activation of human peripheral blood mononuclear cells in vitro with OKT3 followed by low concentrations of IL-2 and TNF (*22*). This led to a 1500-fold enhancement of LAK induction and increased lytic activity for fresh primary lung tumor targets compared with lysis generated by optimal concentrations of IL-2 alone in long-term cultures of cytotoxic lymphocytes.

The antitumor effect of combining anti-CD3 with low-dose IL-2 and TNF was investigated in vivo in mouse models with established melanoma and sarcoma pulmonary metastases (*29*). Our results demonstrated that the administration of a single 5-μg dose of anti-CD3 followed by low-dose IL-2 with TNF potentiated the antitumor effect over high-dose IL-2 alone, IL-2 plus TNF, or anti-CD3 with IL-2 alone. Reduction of metastases (>80%) using the three agents was equal to or exceeded that achieved by ninefold higher concentrations of IL-2 in three tumor models. Using all three agents in combination also significantly prolonged survival and resulted in over 60% of mice achieving long-term survival (>120 days). This was superior to single agents or to other combinations with the three agents causing a synergistic rather than additive effect. These results in vitro and in vivo indicated that the sequential use of anti-CD3, IL-2, and TNF-α for LAK induction and maintenance potentiates antitumor activity, and suggests novel immunotherapeutic manipulations for the treatment of advanced cancer.

CLINICAL STUDIES IN NSCLC

The laboratory results provided the basis for two phase I trials using low-dose IL-2 in conjunction with other immunomodulators in patients with NSCLC. For 1 year beginning in April 1988, we conducted our first phase I clinical trial using TNF administered simultaneously with IL-2 in patients with stage IIIb or IV NSCLC (*30*). Sixteen patients received a continuous 24-hour IV infusion of 6×10^6 IU/m^2 of IL-2 and a simultaneous daily intramuscular dose of TNF (25–100 μg/m^2) for 5 consecutive days (levels I–III). These doses of each biologic when used alone were ineffective in previous trials. A total of 58 cycles of therapy was administered. Treatments were given at 3-week intervals. Common side effects included fever, local skin reaction at the TNF injection site, pancytopenia, and general malaise; all these reactions were reversible within 48 hours after cessation of therapy. Thrombocytopenia (<50K/μL) was the dose-limiting toxicity and established 50 μg/m^2 per day of TNF with IL-2 as the maximum tolerated dose.

Twelve patients in this group of treated patients received more than two cycles of therapy and were evaluable for response (Table 1). Measurable tumor regression occurred in five patients. One patient had a partial response, experiencing a 10-month regression of all pulmonary metastases. In addition, four minor responses (25–50% regression) occurred. Seven patients who had progressive disease before entry into the protocol had radiographic stabilization of disease during therapy before progression (median, 12 weeks; range, 4–16 weeks). The remaining patients had progressive disease after two cycles of therapy. The median survival for patients in this trial was 30 weeks, with 1- and 2-year actuarial survival estimates of 30% and 18%.

As a result of this investigation, a second phase I study was initiated in May 1989 using low-dose OKT3 followed by IL-2 (6×10^6 IU/m^2 per 24-hour continuous infusion) with TNF (25 mg/m^2, daily IM) as given for 5 days in the previous trial. The primary objective of this second trial is to determine an optimal biologic dose of these agents that will yield the highest level of in vitro lytic activity for future phase II trials. To date, 16 patients have received an IV bolus of OKT3 over 2 days, followed by the same regimen of IL-2 and TNF. All patients had histologically proven stage IIIB or stage IV NSCLC refractory to surgical resection or to prior therapy, and a Zubrod performance status of 1 or less. Performance status and previous therapy for each of these patients are listed in Table 2. Patients received escalating doses of OKT3. Headaches, fever, and chills were the side effects related to OKT3 administration. Side effects related to IL-2 and TNF administration were similar to those observed in the first study. One complete response (regression of a chemotherapy- and radiation-resistant endobronchial tumor) and two minor responses have been observed in this ongoing trial.

Lymphocytes and sera were obtained from all patients at regular time intervals during their therapy. The genera-

Table 1. Results of immunotherapy responses in patients with advanced NSCLC

Dose level	Dose			No. of patients treated	No. of patients evaluable	Complete response	Partial response	Minor response
	OKT3, $\mu g/m^2$	IL-2, $x10^{-6}$ IU/m^2	TNF, $\mu g/m^2$					
I	–	6	25	7	6	0	1	2
II	–	6	50	7	5	0	0	1
III	–	6	100	2	1	0	0	1
IV	27	6	25	3	2	0	0	0
V*	27	6	25	7	2	0	0	1
VI	54	6	25	3	2	0	0	I
VII	108	6	25	3	2	1	0	0
Total				32	20	1	1	6

*Patients given a second consecutive week of IL-2 plus TNF.

Table 2. Patient population: Clinical characteristics and cytolytic activity

Patient	Dose level	Prior therapy*	Performance status	No. of cycles	LAK activity, LU†
I	IV	C,R	0	3	153.9 ± 104.8
2	IV	None	0	2	42.9 ± 4.2
3	IV	C	1	1	43.7
4	V	None	0	2	15.6 ± 12.5
5	V	C	1	2	13.1 ± 8.4
6	V	C,R	0	I	33.2
7	V	None	0	I	3.3
8	V	C	0	1	14.6
9	V	None	0	1	4.0
10	V	C	0	1	5.2
11	VI	C	0	2	49.2 ± 19.1
12	VI	C,R	0	I	56.2
13	VI	None	1	2	85.8 ± 7.5
14	VII	S,C,R	0	4	32.3 ± 4.6
15	VII	None	0	2	36.0
16	VII	None	0	1	50.4

*S = surgery, C = chemotherapy, R = radiation.
†Mean ± SE.

tion of LAK activity was monitored during each cycle of therapy by in vitro lysis of Raji targets in a standard 4-hour ^{51}Cr-release assay. All patients generated varying levels of LAK activity. However, higher levels of TNF did not result in statistically significantly higher lytic activities or clinical responses in the first trial (levels I–III, Fig. 1). Increased lysis over pretreatment levels of available autologous tumor targets [range, 70–4150 lytic units (LU)] was demonstrated for four patients.

OKT3 alone induced LAK activity in 11 of the 15 patients (range, 0.5–96.4 LU; mean, 21.4 LU) over baseline values. Endogenous production of TNF (measured by ELISA) in 8 patients was observed (mean, 750 pg/mL). Administration of IL-2 + TNF produced further increases in LAK activity (range, 3.3–361.3 LU) compared with pretreatment levels. Mean generated LAK activity for each patient is shown in Table 2. Optimum biologic activity reflected as the maximum LAK activity was achieved at dose level VI (Fig. 1). This was significantly increased ($P < .05$, two-tailed) over those levels in the first trial without OKT3 (level VI vs. II, 72.6 ± 10.9 vs. 33.0 ± 7.1 LU, respectively). Phenotypic analysis of circulating lymphocytes from both trials revealed increases in TAC expression and in $CD3^+$, $CD16^+$, and $CD56^+$ populations following completion of therapy, with no change in $CD4^+$ or $CD8^+$ populations.

SUMMARY

We initiated our clinical trials using low doses of OKT3, IL-2, and TNF because of efficacy in our previous in vitro

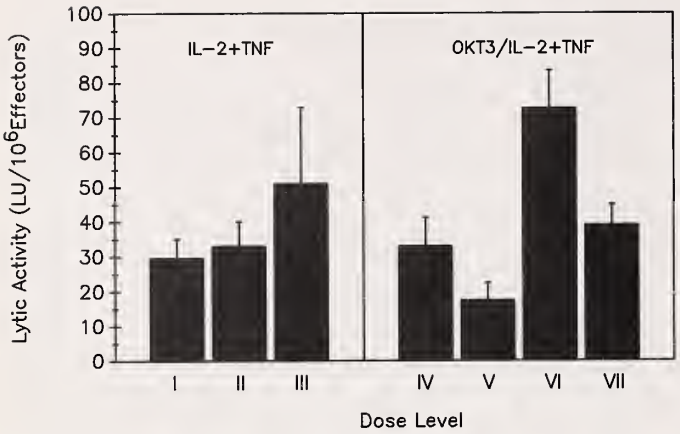

Fig. 1 LAK activity (LU) generated in vivo at the 7 dose levels of therapy. Activity was measured by the in vitro lysis of Raji targets (natural killer-resistant) in a 4-hour ^{51}Cr release assay. Activity was calculated as the inverse number of effectors required to achieve 30% specific lysis $\times$ 10^6.

and animal model experiments. In the 32 patients entered into these studies, one complete, one partial, and six minor responses were seen, with a median survival rate of 30% from the first study. The demonstration of antitumor activity by these and other combination immunotherapy regimens suggests they should be evaluated as adjuvant therapy in patients with early-stage NSCLC.

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Immunologic Effector Cells in Head and Neck Cancer

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ABSTRACT—Freshly isolated tumor-infiltrating lymphocytes (TIL) and lymph node lymphocytes (LNL) in patients with head and neck cancer (HNC) often have low or undetectable functional responses. Because impaired ability of these cells to produce cytokines could be responsible for their functional incompetence, spontaneous and in vitro-induced production of interleukin-2 (IL2), interleukin-1 beta (IL-1β), tumor necrosis factor-alpha (TNF-α), and interferon gamma (IFN-γ) by TIL, LNL from tumor-free as well as tumor-involved lymph nodes (LN), and peripheral blood lymphocytes (PBL) were measured. Although TIL or PBL of patients with HNC produced IL-1β and TNF-α spontaneously or after in vitro activation, LNL did not produce measurable levels of these cytokines. LNL also produced lower levels of IFN-γ than PBL. In situ hybridization for cytokine mRNA performed with tumor tissues, and LN of patients with HNC showed that TIL as well as LNL localized in the immediate proximity of the tumor were activated, as evidenced by the expression of mRNA for IL2, IFN-γ, IL-1β, TNF-α, and both α- and β-chains of the IL2 receptor. In addition, many LNL located next to the tumor expressed mRNA for transforming growth factor-beta (TGF-β). In contrast, LNL not adjacent to the tumor in involved LN, as well as those in tumor-uninvolved LN, did not express mRNA for cytokines or IL2 receptor. These data suggest that TIL and LNL in tumor-involved LN of patients with HNC are activated by the tumor to produce mRNA for various cytokines including TGF-β, which may be responsible for the down-regulation of loco-regional immune responses. [J Natl Cancer Inst Monogr 13:203–208, 1992]

Human head and neck cancers (HNC) are generally well infiltrated with mononuclear cells (MNC), which accumulate at the tumor margins, in the stroma, and even within the tumor parenchyma (*1,2*). Immunohistology with monoclonal antibodies specific for surface antigens on MNC has indicated that T lymphocytes are a major component of tumor-infiltrating lymphocytes (TIL), that $CD8^+$ cells preferentially localize to the tumor parenchyma, and that $CD4^+$ cells predominate in the tumor stroma (*3*). It has been suggested that both the intensity and the composition of these infiltrates are important prognostic parameters in HNC and that they may positively correlate with disease-free survival (*4,5*). These observations imply that MNC infiltrating the tumor play an active role in its progression and that the host's ability to control tumor growth may, in part, depend on functional competence of these cells. Similarly, immunocompetence of lymph node lymphocytes (LNL) has been thought to be important in host-tumor interactions, especially because the cervical lymph nodes are often an initial site of metastasis in HNC (*6*).

Newer methods for quantitative recovery of TIL and LNL from tissues (*7*) have facilitated functional and phenotypic studies of freshly isolated cells (*8*). Phenotypically, fresh TIL or LNL represent mixtures of $CD8^+$ and $CD4^+$ T lymphocytes, many of which are in an activated state, as judged by the expression of HLA-DR or CD25 antigens on the cell surface (*4,8*). Cells with the suppressor phenotype, $CD8^+CD11b^+$ (*9*), although present (generally $<10\%$), are not increased in proportion as compared with the peripheral blood lymphocytes (PBL) (*8*). Functionally, freshly isolated TIL or LNL are completely or partially inhibited in proliferative as well as cytotoxic antitumor responses (*8,10*). The capacity of cells in these populations to develop into lymphokine-activated killer (LAK) cells is also impaired in comparison to that of autologous PBL (*11*). Although the degree of unresponsiveness is variable among TIL or LNL preparations obtained from different patients with HNC, immunosuppression of these cells can be consistently demonstrated in in vitro assays (*12*).

Our hypothesis has been that blunted loco-regional responses of lymphocytes in patients with HNC are due to tumor-induced immunosuppression. Indeed, human solid tumors, including HNC, are known to produce a variety of factors that can inhibit immune responses of lymphoid cells (reviewed in *13*). However, activation of lymphoid regulatory cells among TIL or LNL could also contribute to local immunosuppression, as could a lack or paucity of cytokines in the tumor microenvironment. In this study, abilities of TIL or LNL to express mRNA for cytokines in situ or to produce cytokines were examined. We found that TIL in HNC responded in situ to the tumor by the expression of cytokine genes, mRNA for activation markers, and cytokine production. In contrast, LNL from tumor-involved lymph nodes (LN) were deficient in these responses except for a small number of LNL bordering the tumor. Many of the LNL situated next to the nests of

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tumor cells also expressed mRNA for transforming growth factor-beta (TGF-β).

MATERIALS AND METHODS

Patients

Twelve patients with HNC (eight male and four female) at ages from 53 to 75 years were included in this study. None of the patients was previously treated. All underwent surgical resections of primary tumor and/or modified radical neck dissections. The primary tumor sites and numbers of tumors were larynx = 2; hypopharynx = 4; oral cavity = 2; oropharynx = 4. Tumors were moderately or moderately well-differentiated carcinomas. Eight tumors were stage III and four were stage IV. All the patients had at least one metastatic node, as determined by histologic examination. Whenever possible, tumor-involved lymph nodes (I-LN) as well as tumor-noninvolved (NI-LN) were obtained from the same patients (8/12 cases).

Control Tissues

Samples of fresh human tonsils (n = 5) and reactive lymph nodes (n = 5) were obtained from the Surgical Pathology Laboratory at Presbyterian University Hospital.

PBL, TIL, or LNL Isolation

PBL were obtained from the venous blood of patients prior to surgery and isolated by Ficoll-Hypaque centrifugation. Monocytes were removed by adherence to plastic for 1 hour in RPMI-1640 containing 10% (vol/vol) fetal calf serum (FCS) at 37 °C in 5% CO_2. Lymphocytes were then washed and resuspended in tissue culture medium (TCM) consisting of MEM (Gibco, Grand Island, N.Y.) supplemented with 2 m*M* L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 15% (vol/vol) FCS (all from Gibco).

Samples of cervical LN were obtained from the resected specimens by a pathologist. Half of each LN was used for histopathology and the other half for cell recovery. Lymph nodes were disaggregated by passage through a wire screen. Suspensions containing tumor cells and MNC were separated on differential Ficoll-Hypaque gradients as described (*14*). The presence of tumor cells in each LN was confirmed by histopathology. Recovered cells were washed twice in TCM and checked for viability using a trypan blue dye.

TIL were prepared by controlled enzymatic digestion of tumor tissues as described earlier (*7,14*). Following separation on differential Ficoll-Hypaque gradients, TIL were harvested, washed, and checked for purity on May-Grunwald-Giemsa-stained smears and for viability with a trypan blue dye. TIL were cryopreserved using a Cryomed freezer (Mt. Clemens, Mich.) and stored in liquid N_2 vapors until needed for assays.

Cytokine Production and Assays

Freshly obtained or cryopreserved and defrosted TIL, LNL, or PBL were tested for spontaneous and in vitro-induced production of IFN-γ, TNF-α, IL-1β, and IL2. Cells were plated in wells of 96-well plates (1×10^5/well). For TNF-α and IL-1β production, 1μg of LPS (*E. coli* 026:B6, Sigma Chemical Co., St. Louis, Mo.) was added to each well, and plates were incubated for 24 hours at 37 °C in 5% CO_2. For IL2 and IFN-γ production, 2 μg of PHA-P (Sigma Chemical Co.) was added to each well in a final volume of 200 μL of TCM. Plates were incubated for 48 hours at 37 °C in 5% CO_2. Following incubation, plates were centrifuged at 600*g* for 3 minutes to sediment the cells, and 150 μL aliquots of cell-free supernatants were harvested and used for cytokine immunoassays. IFN-γ was measured by the RIA (Centocor, Philadelphia, Pa.); IL-1β and TNF-α were assayed by ELISA as described by us earlier (*15*); and IL2 was assayed by ELISA using reagents purchased from Collaborative Research, Inc. (Bedford, Mass.). All cytokine assays were calibrated against the World Health Organization (WHO) cytokine standards. Control lymphocyte supernatants were prepared by inducing normal human MNC with LPS or PHA-P, aliquoted into 1-mL vials, frozen at −80 °C, and included in every assay at different concentrations to monitor day-to-day variability.

In Situ Hybridization

Tumor or lymph node biopsies from patients with HNC as well as samples of hyperplastic human lymph nodes or tonsils were embedded in OCT compound (Miles Laboratories, Naperville, Ill.) and snap-frozen in liquid N_2 within 45 minutes after surgical removal. Tissue samples were stored in OCT at −80 °C.

Prior to sectioning, tissue blocks were warmed in a cryostat (−20 °C). Tissue sections (10 μm) were cut and mounted on gelatin-coated slides. Following air drying, tissue sections were fixed with 4% (vol/vol) paraformaldehyde (Sigma Chemical Co.) in phosphate-buffered saline (PBS) for 10 minutes and washed with 70% ethanol in diethyl-pyrocarbonate (DEP) -treated H_2O. All reagents used for in situ hybridization were prepared with DEP-treated H_2O. Next, the sections were rehydrated in PBS and 50 m*M* $MgCl_2$ for 15 minutes and then washed in 200 m*M* TRIS-HCl-glycine buffer, pH 7.4, for 15 minutes. The specimens were next acetylated with 0.25% acetic anhydride (Sigma Chemical Co.) in 0.1 *M* triethanolamine and 2×SSC (1×SSC = 0.15*M* NaCl/0.015 *M* sodium citrate) buffer for 15 minutes. Finally, the slides were washed in 2×SSC buffer for 10 minutes before the prehybridization step. Control slides treated as above were incubated with a solution containing 100 μg/mL DNAse-free ribonuclease A and 10 U/mL ribonuclease T1.

Positive and negative controls for in situ hybridization were obtained using human peripheral blood mononuclear cells (PBMNC) activated with human recombinant IL2 (rIL2) or resting PBMNC, respectively.

The cDNA probes were isolated from plasmids with the respective restriction enzymes exactly as described earlier (*16*). The following cDNA fragments were prepared: IL2 (900 bp), TNF-α (800 bp), and IFN-γ (950 bp), all from Dr. M. Palladino, Genentech Inc., South San Francisco, Calif.; IL2 receptor β-chain p70 (1.05 Kilobases, kb) from Dr. T. Taniguchi (Institute for Molecular and Cellular Biology, Osaka University, Japan); IL2 receptor α-chain p55 (937 bp) from W. Leonard, National Institutes of Health; the β-tubulin (DB-1) cDNA fragment (750 bp) from Dr. N. Cowan, N.Y.U. Medical Center; and the 18S cDNA fragment (3000 bp) from C. Milkarek, University of Pittsburgh. The pUC-9 plasmid cDNA was cut with Pst 1 restriction enzyme to obtain fragments of about 2700 bp for use as a negative control. These cDNA fragments were labeled with ^{35}[S]dATP (NEN-DuPont, Boston, Mass.) using a kit for random hexanucleotide priming (Bethesda Research Laboratories, Bethesda, Md.) according to the method described by Feinberg and Vogelstein (*17*). The specific activity of the probes used for in situ hybridization assays was at least 1×10^8 cpm/μg of labeled fragment.

After rehydration of tissues as described above, tissue sections and cytologic specimens were prepared for hybridization by flooding them with 50% formamide (Bethesda Research Laboratories) in 2×SSC buffer and warming up to 70 °C. The hybridization mixture contained 10% dextran sulfate, 2×SSC, 500 μg of tRNA per mL, 0.2 mg of BSA per mL, and 10 m*M* of DDT (dithiothreitol) in DEP-treated water. Equal volumes of the radiolabeled cDNA probe resuspended in 100% formamide and denaturated by boiling for 10 minutes and of the hybridization mixture were placed onto the specimens, covered with parafilm, covered with a coverslip, and sealed with rubber cement. The specimens were hybridized overnight in a humidified chamber at 45 °C. After hybridization, the specimens were extensively washed in 50% formamide in 2×SSC for 30 minutes at 42 °C and then with 50% formamide in 1×SSC. The dehydrated slides were dipped in 1:1 mixture of 0.6 *M* ammonium acetate-NTB-2 Kodak autoradiographic emulsion melted at 42 °C, and exposed in black boxes for 1 week to 10 days. The slides were then developed in Kodak D19 developer for 5 minutes, rinsed in water for 1 minute, and treated with Kodak Fixer A for 5 minutes. The specimens were finally counterstained with hematoxylin, mounted in permount, and evaluated in a Leitz microscope.

Microscopic Examination and Cell Counts

The experimental conditions used for in situ hybridization allowed us to obtain very low background and to increase the specificity of the assay. Cells considered positive for cytokine mRNA contained at least 25 grains/cell by autoradiography. Mononuclear cells positive for mRNA for cytokines were counted in five representative high-power fields (HPF) at magnification of ×400, and the results were expressed as the number of positive cells per one HPF. In cryostat sections of tumor tissues, it was generally possible to distinguish between mononuclear cell infiltrates in the stroma and tumor cells. Serial cryostat sections, each hybridized with a different probe, were used to count cells positive for different cytokine mRNA.

Statistical Analysis

The significance of differences between cytokine levels produced by TIL, LNL, or PBL was calculated using Student's *t*-test, and the *P* value $< .05$ was considered significant.

RESULTS

To evaluate functional responses of fresh TIL, LNL, and PBL in patients with HNC, the ability of these cells to produce cytokines spontaneously or in response to exogenous activators was measured. Freshly harvested or fresh-frozen cells were incubated in TCM or in TCM supplemented with stimulating agents, and spontaneous or induced production of TNF-α, IL-1β, IFN-γ, or IL2 was measured by immunoassays in the cell supernatants. As shown in Table 1, tumor-involved or noninvolved LNL did not produce measurable levels of TNF-α or IL-1β in vitro. In contrast, PBL obtained from patients with HNC spontaneously produced significantly more ($P < .001$) TNF-α and IL-1β than PBL of normal individuals. TIL spontaneously released TNF-α and IL-1β at levels that were not significantly different from those of normal PBL; however, induced in vitro production of these

Table 1. Spontaneous and induced in vitro production of IL-1β and TNF-α by TIL, LNL, or PBL obtained from patients with head and neck cancer*

		IL-1β, pg/mL		TNF-α, pg/mL	
Lymphocytes	n	Spontaneous	Induced	Spontaneous	Induced
TIL	7	47 ± 26	39 ± 17†	36 ± 19	70 ± 35†
LN-1	11	0	0	0	0
LN-NI	10	0	0	0	0
Patient PBL	11	1036 ± 525†	1472 ± 824	370 ± 173†	610 ± 212
Normal PBL	30	32 ± 7	4600 ± 473	12 ± 3.5	1550 ± 128

*Fresh or fresh-frozen lymphocytes were incubated in TCM (spontaneous) or in TCM plus LPS (induced) for 24 hours as described in Materials and Methods. Culture supernatants were harvested and assayed for cytokines. Data are means ± SEM.

†$P < .001$ vs. normal PBL.

cytokines by TIL was much lower ($P<.001$) than that of normal or patient PBL (Table 1).

The data shown in Table 2 indicate that TIL or LNL from patients with HNC did not spontaneously release IFN-γ. Induced production of IFN-γ by patients' cells was lower than that of normal PBL ($P<.01$). On the other hand, PHA-P-induced IL2 production was normal in LNL and TIL.

Overall, considerable differences were observed between patients' lymphocytes from different compartments in the ability to synthesize cytokines, with LNL being the least responsive, and PBL characterized by the higher-than-normal spontaneous release of TNF-α and IL-1β. TIL differed from LNL in that they produced variable, but nearly always detectable, levels of all four cytokines.

To further examine the activation state of TIL and LNL in patients with HNC, in situ hybridization for cytokine mRNA was performed. As shown in Fig. 1, activated MNC expressing mRNA for cytokines and IL2R-α or IL2R-β were present in the tumor stroma (Fig. 1, A and B). In contrast, most LNL in tumor-involved LN were not activated, and only those cells in an immediate proximity to the tumor contained mRNA for cytokines IL2 (Fig. 1, C), IFN-γ, TNF-α, and IL-1β as well as for IL2R-α or IL2R-β. In addition, numerous LNL located next to the tumor contained a message for TGF-β (Fig. 1, D). We were unable to detect mRNA for TGF-β in tumor cells in situ.

As control, normal reactive human LN and tonsils were also examined by in situ hybridization for the presence of MNC expressing cytokine genes. As shown in Fig. 2 and determined by microscopic cell counts of sections obtained from five LN and five tonsil specimens (data not shown), normal reactive human LN contained few cells positive for cytokine genes.

These data indicate that in HNC, the tumor is capable of inducing activation of TIL or LNL and that some of these activated MNC might contribute to local immunosuppression by the production of soluble immunoregulatory factors such as TGF-β.

DISCUSSION

In HNC, the cervical LN are frequently the initial site of metastasis (*6*). For this reason, immune responses of LNL, especially those derived from tumor-involved LN, have been carefully evaluated recently (*8,12,18*). Cells obtained from such LN appear to be unresponsive in vitro to activation stimuli. Among explanations that have been advanced for this deficiency in loco-regional immunocompetence, two deserve attention.

First, inability or decreased ability of LNL to produce cytokines, which are necessary for an immune response to proceed, may be responsible for local immunosuppression in patients with HNC. We have examined this alternative in the experiments described here and observed that LNL and TIL closest to the tumor are activated and express mRNA for cytokines. In comparison to normal reactive human LN, tumor-involved LN from patients with HNC contained more MNC positive for cytokine genes. In situ hybridization experiments demonstrated that MNC expressing cytokine genes were restricted in their localization to the areas bordering the tumor in tumor-involved lymph nodes, and in the tumor stroma, MNC were situated nearest to tumor cells. Like normal hyperplastic LN, tumor-uninvolved LN in patients with HNC contained few MNC-expressing cytokine genes. These observations suggested that tumor cells could induce activation of LNL and TIL in situ. Recent in vitro experiments in our laboratory confirmed that human lymphoid cells incubated with a squamous cell carcinoma of the head and neck cell line were induced to up-regulate IL2R; to express mRNA for TNF-α, IFN-γ, IL2, and IL2R; to produce TNF-α and IFN-γ; and to proliferate (unpublished data). Thus, it appears that spontaneous production of certain cytokines by TIL, LNL, and PBL obtained from patients with HNC (Tables 1 and 2) may be a function of the tumor presence. In respect to the fact that patients' LNL did not produce measurable levels of TNF-α or IL1-β in our in vitro assays, it is possible that differences in the proportion of activated cells recovered from different sites may contribute to this observed deficit.

Second, it has been suggested that suppression of antigen-reactive T lymphocytes by regulatory cells may be responsible for inadequate immune responses of LNL or TIL (*19*). Such regulatory or suppressor cells activated in vivo in the tumor milieu could down-regulate antitumor responses of other lymphocytes in the microenvironment. However, in a recent study, we failed to identify "suppressor" T lymphocytes by their phenotypic or functional

Table 2. Spontaneous and induced in vitro production of IL2 and IFN-γ by TIL, LNL, or PBL obtained from patients with head and neck cancer*

		IL2, U/mL			IFN-γ, U/mL	
Lymphocytes	n	Spontaneous	Induced	n	Spontaneous	Induced
TIL	6	2.3 ± 1.6	43 ± 18	6	0	46 ± 23†
I-LN	9	1.7 ± 0.8	65 ± 12	9	0	35 ± 8†
NI-LN	9	2.0 ± 0.8	66 ± 11	9	0	20 ± 10†
Patient PBL	10	2.5 ± 1.8	20 ± 6.8	12	2.1 ± 2.3	76 ± 40†
Normal PBL	33	0.6 ± 0.2	17.3 ± 2.3	37	4.6 ± 1.2	267 ± 31

*Fresh or fresh-frozen lymphocytes were incubated in TCM (spontaneous) or in TCM plus PHA-P (induced) for 48 hours as described in Materials and Methods. Culture supernatants were harvested and assayed for cytokines. Data are means ± SEM.

†$P<.01$ vs. normal PBL.

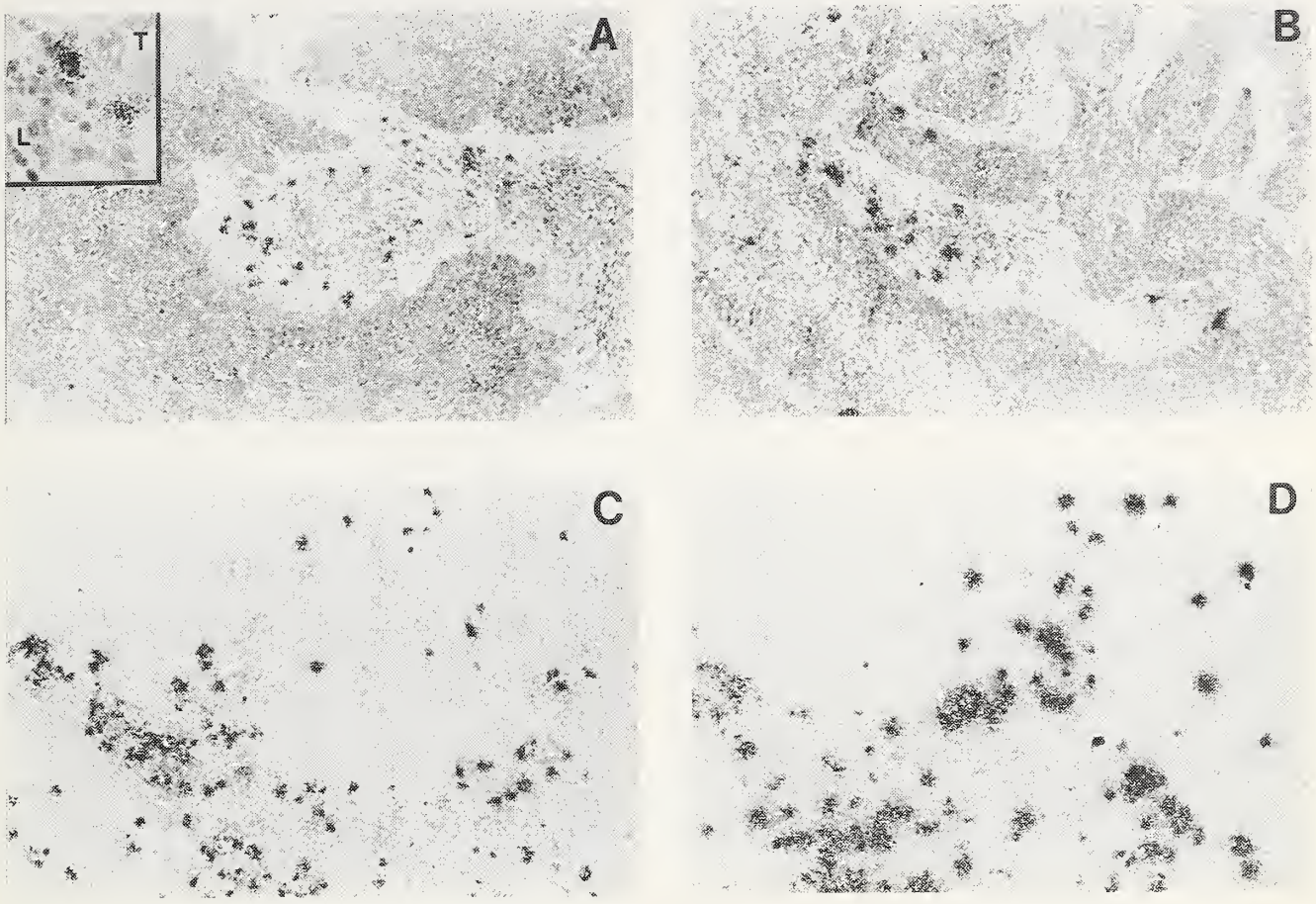

Fig. 1. In situ hybridization with ^{35}[S]-labeled cDNA probes for expression of mRNA for cytokines or IL2 receptor in tumor tissues (A and B) or tumor-involved LN from patients with HNC. A, cells positive for TNF-α mRNA are shown in the tumor stroma ($\times$100). At higher magnification ($\times$400), it is possible to determine that MNC in the tumor stroma are labeled (*see inset*). B, Cells positive for IL2 receptor α-chain mRNA in the stroma ($\times$100). C and D, Serial sections of a tumor-involved LN showing the presence of cells positive for IL2 mRNA (C) and of numerous cells positive for TGF-β (D) around the tumor in the remaining lymphoid tissue. Original $\times$100.

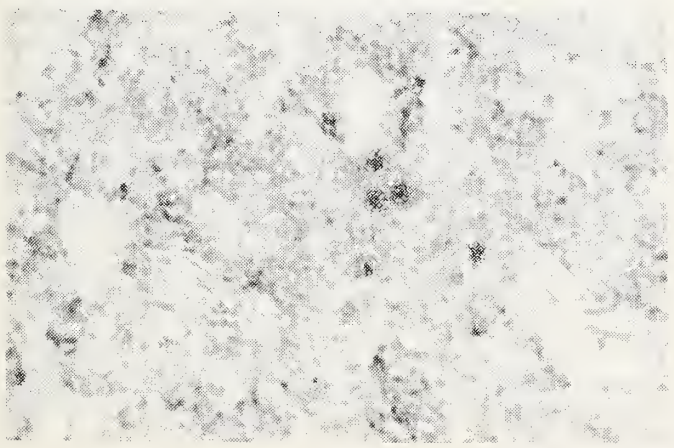

Fig. 2. In situ hybridization with ^{35}[S]-labeled cDNA probe for expression of mRNA for IL2 in a frozen section of human reactive LN. Original $\times$250.

properties among LNL of patients with HNC (*12*). In situ hybridization, which allows for the localization and identification of activated MNC in tumor-involved LN or tumor stroma, provided evidence that such regulatory cells may indeed be induced in the tumor-involved microenvironment. These cells producing TGF-β in response to tumor-induced signals might down-regulate functional activities of other LNL distant from the tumor. Even LNL in the neighboring tumor-free LN may be so down-regulated. It is possible, for example, that the tumor produces TGF-β, which can bind to receptors on TIL or LNL, induce expression of TGF-β genes, and initiate an autocrine TGF-β mechanism responsible for local immunosuppression.

Our results suggest that despite the ability of the tumor to activate MNC in situ, immune responses in tumor-involved LN of patients with HNC may be depressed. Immunosuppression observed in in vitro functional assays with TIL or LNL (*12,13*) could be a result of activation of

MNC able to produce TGF-β. In vitro, exogenous cytokines, specifically high doses of rIL2 (100-1000 Cetus U/mL), reverse this immunologic unresponsiveness of LNL (*8*), and IL2-activated T lymphocytes derived from LN of patients with HNC are a good source of broadly reactive antitumor effector cells (*20*). Furthermore, in patients with inoperable HNC treated with intratumoral rIL2 in an ongoing Phase I ECOG clinical trial, significant up-regulation of local antitumor effector function has been demonstrated. A better understanding of interactions between tumor and immune effector cells is essential for designing novel approaches to therapy of HNC in the future.

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